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(54) Title: INTEGRATED NUCLEIC ACID HYBRIDIZATION DEVICES

(57) Abstract: The invention provides devices and methods for enhanced and selective association or binding between biological materials, such as nucleic acids, e.g., DNA or RNA, or polypeptides, and an immobilized oligonucleotide probe. In one embodiment, the invention provides an association device comprising a plurality of nucleic acid probes or polypeptide probes or a combination thereof linked to a solid substrate; wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end opening to the external surface to allow passage of fluid into a pore, and wherein the pore surfaces comprise an association surface; wherein the association surface comprises a charged surface comprising net positive (cationic) charge density under conditions comprising a pH lower than the pI of the association surface. Methods for making these hybridization/association devices are also provided. For example, the devices and methods of the invention can be used in nucleic acid-based diagnostic tests. The devices and methods of the invention can be used, e.g., for detecting the association of a nucleic acid in a sample to a nucleic acid probe or purifying a nucleic acid from a sample.

INTEGRATED NUCLEIC ACID HYBRIDIZATION DEVICES

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application is a Continuation-In-Part (CIP) of U.S. Patent Applications Serial No. ("USSN") 09/522,240, filed March 09, 2000; which is a CIP of USSN 09/513,063, filed on February 24, 2000; which is a continuation of USSN 08/749,967, filed on November 14, 1996 which claims benefit of priority under 35 USC §119(e) of provisional application serial no. 60/006,696, filed November 14, 1995. This aforementioned applications are explicitly incorporated herein by reference in their entirety and for all purposes.

Field of Invention

The present invention generally relates to the fields of molecular biology and diagnostic medicine. In particular, the invention relates to "association" devices and methods for enhanced and selective association or binding between nucleic acids, such as DNA or RNA, or polypeptides, and an immobilized oligonucleotide or polypeptide probe. In one embodiment it relates to "association" devices that can be used for, e.g., nucleic acid-based diagnostic tests, isolation of nucleic acids or polypeptides, and the like.

Background of the Invention

The current demand for nucleic acid sequence analysis has spurred the rapid development of new technologies which will enable such information to be collected with increasing efficiency. In particular, the use of massively parallel, array-based hybridization detection devices are being investigated. Modern concepts in microelectronic engineering, nanotechnology, optical physics and information processing are being combined to develop devices which allow the collection and assimilation of large amounts of data in extremely short time frames. Ultimately, the employment of such devices may replace cumbersome molecular biological protocols which have traditionally been used for the generation of data for a variety of purposes, including but not limited to: 1) genome analysis, 2) mutation detection, 3) pathogen detection, 4) RNA analysis and 5) nucleotide sequence determination.

The biochemical basis for the ability to use a hybridization assay for any of the above mentioned reasons, either by modern or traditional methodologies, resides, of

course, in the complementary nature of nucleic acids, originally elucidated by Watson and Crick in the early 1950s. Since then, the exploitation, refinement and further characterization of the specific hydrogen bonding patterns of complementary nucleic acid strands has led to a huge volume of information, and has profoundly affected the paradigm of medicine and biology.

The ability to hybridize nucleic acids with adequate sensitivity and selectivity is strongly dependent on a number of physical chemistry considerations and has been a subject of considerable attention. Of primary importance, is the electrostatic nature of nucleic acids and the need for bulk solution cations to screen the negative charges of the phosphate groups on the backbone, allowing hydrogen bonding to occur between the two negatively charged polyelectrolytes. Electrostatics play a critical role *in vivo* also, being a primary determinant in cellular functions such as replication, recombination, transcription, chromatin structure, packing and ligand binding.

Until recently, the bulk of hybridization analysis has been performed on nitrocellulose, nylon or other membranous type solid supports. The method of attachment of macromolecules to these types of support is at best semi-specific, involving non-covalent capture in microporous channels. It has recently been demonstrated that the covalent tethering of probe molecules to other types of solid supports may enhance hybridization association binding constants by up to two orders of magnitude. For these reasons and for issues of compatibility with developing hybridization detection devices, covalent and other non-traditional means of surface immobilization of nucleic acids are becoming more commonplace. Although a great deal is known about the chemical interactions of polymers at solid interfaces, there is a paucity of information regarding the biophysical interactions of nucleic acids at such interfaces as compared with solution state physical data.

In the prior art, the parameters affecting the hybridization of nucleic acids has been traditionally characterized from solution state experiments or from experiments performed on membranous type supports in which target molecules are non-specifically absorbed or sequestered. Until the present invention, the active participation of a substrate

material upon which probes are specifically tethered in the hybridization reaction has not been considered.

The closest equivalent to the proposed invention is the use of DNA probes using modified backbones such as peptide nucleic acids (PNAs) to alter the ionic character of binding to targets. These have been investigated primarily as gene-based therapeutic agents and have not been used on substrate surfaces compatible with arrayed detection methodologies. In any case, the principle is completely different and is not readily compatible with large scale combinatorial approaches.

Summary of the Invention

The present invention provides devices and methods to improve the specificity and kinetics of the association, e.g. hybridization, of nucleic acids in solution to immobilized nucleic acids (e.g., probes). The probes can be immobilized on any surface in any configuration, such as, e.g., microtiter well plates, array-based hybridization detection devices, porous substrates (beads, membranes), and the like. The invention provides significant increases in the kinetics, sensitivity and discrimination power of nucleic acid-based (e.g., DNA and RNA-based) and polypeptide-based biosensors and related hybridization techniques.

Further, the present invention is useful in modern molecular techniques using large array-based strategies. In these strategies, oligonucleotide probe molecules are bound (covalently or non-covalently attached) to a device-compatible substrate, often in a miniaturized format. The exploitation of the "smart" or "tunable" surfaces of the invention upon which to perform association or hybridization is imminently useful in maximizing the information output of modern nucleic acid detection or isolation devices. The present invention for the first time uses the active participation of a substrate material upon which oligonucleotide probes are specifically tethered in nucleic acid association/ hybridization reactions.

Accordingly, the invention provides association devices for the detection and/or isolation of nucleic acids in a test sample. The association can by convention Watson-Crick base pairing, Hoogsteen-based double or triple helix formation, reverse Hoogsteen

hydrogen bonding interactions, and the like, as discussed in detail below. Thus, one embodiment of the present invention provides an association/hybridization device for detecting a target area in a nucleic acid, e.g., a DNA, a cDNA or a RNA, or analogs or mimetics thereof.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention an association/ hybridization device comprising an oligonucleotide probe, and a solid substrate, said solid substrate having a support or solid surface with a "tunable" positive, neutral or negative net electrostatic field. The "tunable" support surface comprises an association/ hybridization surface accessible for linking the oligonucleotide probe to the solid substrate or association surface. In one embodiment, the oligonucleotide probe can be linked to the association/ hybridization surface of the solid substrate at a distance of no more than about 100 angstroms.

In one specific embodiment the oligonucleotide probe is linked to the hybridization surface by a covalent linkage or a slowly reversible, non-covalent linkage. In alternative embodiments the distance is no more than about 75 angstroms, 60 angstroms, 50 angstroms, 30 angstroms, or about 20 angstroms.

In specific embodiments, the support surface of the device has a negative electrostatic field, i.e., a net negative field density. This can include support surfaces having, e.g., a layer of negatively charged protein film, or any composition which can switch the charge of the electrostatic field in the range of about pH 5 to about pH 8 to about pH 10. The switch can be, e.g., from positive or neutral during association to negative during washing.

Further, the support surface or hybridization surface can be composed of compositions where the electrostatic field (the net charge density of the surface of the device) is more cationic (positive net charge density) during hybridization and more anionic (negative net charge density) during washing. In various embodiments, the support surface or association (e.g., a hybridization) surface of the device comprises a composition having a pI in a range of between about pI 5 to about pI 10. Accordingly, if the pH of the surface of the device is adjusted to a point at least just below (acidic to) the pI of the composition (by, e.g., adjusting the pH of the association/hybridization solution), then the support surface or

association (e.g., hybridization) surface of the device has a net positive (cationic) charge density (i.e., net positive electrostatic charge). This promotes the association of a nucleic acid to the immobilized nucleic acid probe (which is linked to the solid substrate or to the association surface). The test sample is contacted with (e.g., applied to) the association device under these conditions (i.e., that induce the substrate surface or association surface to have a net positive (cationic) charge density).

In other embodiments, for the "wash" phase, the device is adjusted to a point at least just above (basic to) the pI of the composition (by, e.g., adjusting the pH of the association/hybridization solution), then the support surface or association (e.g., hybridization) surface of the device has a net negative (anionic) charge density (i.e., net negative electrostatic charge).

Thus, in various embodiments, the support surface or association surface (e.g., "hybridization surface") can be composed of compositions where the electrostatic field is cationic or neutral at about pH 5 to about pH 6, at about pH 5 to about pH 7, at about pH 5 to about pH 8, at about pH 5 to about pH 9, or at about pH 5 to about pH 10; and negatively charged at about pH 7 to about pH 8, at about pH 7 to about pH 9, or at about pH 7 to about pH 10.

In alternative embodiments the association/hybridization surface can be streptavidin, imidazole, imidazole derivatives, carboxylic acid, histidine, histidine derivatives, citrate or other groups, including those with a pK value near neutrality. For example, as the pI of strepavidin is 5.5; in a device of the invention comprising strepavidin on its solid support surface, the association conditions are adjusted to be below 5.5 (with, however, a practical lower range of about pH 4.5), and wash conditions are adjusted to be above 5.5 (with a practical upper limit of about pH 10).

In one embodiment the association/ hybridization surface is streptavidin and the oligonucleotide probe comprises biotin (e.g., is modified to include biotin); in this example, the oligonucleotide probe is linked to the hybridization surface by non-covalent interaction of the biotin with the streptavidin. Oligonucleotide probes used in the invention ca be conjugated or modified by any first compound having an affinity for a second

compound which is attached (covalently or non-covalently) to the association surface or the solid support surface of the device.

In further embodiments, the association/hybridization surface can be arginine, an arginine derivative, salmine A1, a salmine A1-probe chimera linked to the support surface, any amino acid, an amino acid ester or a mixture of amino acids and/or amino acid esters. Primary amine groups have a pI of 10. Thus, in alternative embodiments, the device can be adjusted to a pH anywhere below pH 10 for association conditions on the solid support surface (e.g., the "association surface") to be a net positive charge density; and pH 10 or higher for wash conditions.

The association/ hybridization surface also can be comprised of two or more different compounds, e.g., those listed herein.

The solid support surface of the device can be any composition. For example, in alternative embodiments the support surface can be metal, polyvinyl, polystyrene, polypropylene or polyester. The association/ hybridization surface can be a carboxylic acid surface placed on the support surface; where the oligonucleotide probe has been modified to include an amino group; and the modified probe is covalently linked to the association/ hybridization surface. For example, in one embodiment the support surface is polystyrene; the association/ hybridization surface is streptavidin; the oligonucleotide probe has been modified to include biotin; and the oligonucleotide probe is linked to the hybridization surface by non-covalent interaction of the biotin with the streptavidin.

In another embodiment the support surface is glass and the oligonucleotide probe and glass form a substrate surface-probe complex by linking the probe to the substrate surface by an epoxysilane linkage to a terminal amine modification, and the substrate surface-probe complex forms an effective association/ hybridization surface.

Another specific embodiment includes a method for detecting single base difference in a target area of a strand of DNA or RNA comprising mixing a nucleic acid with an association/ hybridization device of the invention with immobilized oligonucleotide probes containing (with sequence complementary to) the target area to be detected; allowing sufficient time for the target area to associate/ hybridize to the device; altering the

environment of the oligonucleotide probe and DNA or RNA target area to remove non-hybridized D[redacted]r RNA; and detecting the DNA or RNA hy[redacted]zed to the association/ hybridization device.

The invention provides a method for promoting the association (e.g., hybridization) of a nucleic acid to an immobilized nucleic acid probe comprising the following steps: (a) providing a test sample comprising a nucleic acid; (b) providing a device comprising an oligonucleotide probe linked to a solid substrate; wherein the solid substrate comprises a substrate surface and an association surface; wherein the association surface comprises a composition having a pI in a range of between about pI 5 to about pI 10; and (c) contacting the test sample of step (a) with the association device of step (b) under conditions that induce the substrate surface or association surface to have a net positive (cationic) charge density.

The device can be designed such that the charged surface (e.g., the net positive (cationic) charge density) is close enough to the oligonucleotide probe (the area in which the association/ hybridization with nucleic acid sample will occur) to measurably influence the kinetics or specificity of association. In various embodiments, the distance is between the oligonucleotide probe and the positively charged surface is at or no more than about 100 angstroms, about 50 angstroms, about 30 angstroms, or about 20 angstroms. However, depending on the composition of the association surface (discussed below) and the degree of charge field generated, or desired, the distance can be greater or smaller.

In various embodiments, the substrate surface or the association surface of the device of the invention comprise different compositions (or mixtures thereof). Thus, the device can be designed to have any net pI, and, as it is the pI which determines when the device will have a net positive or a net negative charge field under particular acidic or basic (pH) conditions, the device can be designed to have a net positive or negative charge field under specific pH conditions. Conditions that induce the substrate surface or association surface to have a net positive (cationic) charge density comprise a pH below the pI of the composition. Conditions that induce the substrate surface or association surface to have a net negative (anionic) charge density comprise a pH above the pI of the composition. Thus, by

manipulating the pH of the association/ hybridization conditions, the device has a net positive or a negative charge. Significantly, the device itself substantively influences the kinetics and specificity of association/ hybridization. Furthermore, as the device is "tunable," i.e., can have either a positive or a negative net charge density on its support or association surface, the device of the invention can influence the kinetics and/or specificity of both sample nucleic acid:probe association (as in a hybridization step) and disassociation (as in a washing step).

In the methods of the invention, the association conditions can further comprise no salt or low salt conditions. Low salt conditions comprise any set of conditions wherein the sodium ion (or equivalent ion) concentration is less than seen under physiologic conditions. For example, low salt conditions can comprise less than about 100 mM, about 75 mM, about 50 mM, about 25 mM, about 20 mM, about 15 mM, or about 5 mM, or about 2 mM sodium or equivalent cation. For example, the salt can be sodium chloride, potassium chloride, sodium phosphate, or equivalents. Thus, in alternative embodiments, low salt conditions can comprise about 100 mM, about 50 mM, about 30 mM, or about 20 mM, about 15 mM, or about 5 mM, or about 2 mM sodium phosphate. In one exemplary embodiment, the contacting step is under conditions equivalent to an aqueous solution at about pH 4.5 to about 5.4 comprising about 0.1% to 0.3% detergent and about 2 mM sodium phosphate (typically, this is when the device association surface has a net pI of about pI 5.5 to 6, e.g., when the surface comprises streptavidin). In another embodiment, the substrate surface is pre-treated (before the hybridization step) with an aqueous solution of detergent and neutral polymer. The detergent can be anionic, cationic, zwitterionic, or a combination thereof, e.g., Tween, NP-40, Triton X-100, CHAPS, and the like. For example, the pre-treatment solution can be about 0.1% to about 0.3% detergent and about 2X to about 5X Denhardts's solution.

The methods of invention can be practiced and the device can be used at any temperature. However, one practical advantage of the invention is that it can be practiced at temperatures at or approximately room temperature or about 22°C to 25°C. For example, in the devices and methods of the invention, the kinetics of association between the oligonucleotide probe and the nucleic acid in the test sample are at least ten fold more rapid

than the kinetics of association under conditions wherein the substrate surface or association surface has a neutral or net negative charge density (including conditions at room temperature).

The probes of the invention can comprise any nucleotide, as defined in its broadest sense, including, e.g., synthetic variants of DNA and RNA; single stranded and double stranded probes, linear, circular or "stem-looped" structures; DNA:DNA, RNA:RNA or chimeric structure (e.g., mixed linear, DNA:RNA or other chimerics). The probes of the invention can be of any length; e.g., oligonucleotides used as probes in the devices of the invention can have a length of at least about 11 to about 20 residues in length, or to about 30 residues, 40 residues, 50 residues, 75 residues, 100 residues or greater in length. The device can comprise any number of probes; in one embodiment the device of the invention comprises a plurality of immobilized oligonucleotide probes. Devices with pluralities of immobilized probes have been called "arrays" or "DNA chips." As discussed in detail below, the "tunable" surfaces of the invention can be incorporated into any known "array-based" nucleic acid association/ hybridization device, or combination, or variation thereof.

As described below, the association surface can comprise a variety of compounds or mixture thereof; for example, a composition comprising the association surface can comprise streptavidin, which has a pI of about 5.5. In this embodiment (with streptavidin), the association (e.g., hybridization step) conditions can comprise a pH of below 5.5 to set a net cationic charge density to the device's surface.

Alternatively, the composition can comprise a primary amine group having a pI at about 10; thus, under association conditions comprising a pH of below 10, the net charge density of the surface of the device is negative. Alternatively, the composition can comprise a peptide having a pI at about neutrality or in the range of about pH 6.7 to about pH 10. In various embodiments, the composition comprising a primary amine group can be an amino silane, a polylysine, or an organic polyamine, or equivalents thereof. The peptide can be, e.g., polyhistidine, poly-$(his)_2(gly)_1$, or poly-$(his)_2(asp)_1$, or equivalents thereof. Alternatively, the composition can comprises an organic molecule having a pI at about

neutrality or in the range of about 5 to about 10. The organic molecule can be, e.g., an imidazole derivative, or equivalents thereof.

In practicing the methods and using the devices of the invention, the association of the nucleic acid in the sample to the nucleic acid probe can comprise, e.g., Watson-Crick base pairing, Hoogstein based double or triple helix formation or reverse Hoogsteen hydrogen bonding interactions. Accordingly, the probes of the invention can be either single stranded, double stranded, stem-loop configured, and the like.

The invention also provides methods for detecting association of a nucleic acid in a sample to a nucleic acid probe or purifying a nucleic acid from a sample comprising the following steps: (a) providing a sample comprising a nucleic acid; (b) providing an association device comprising an oligonucleotide probe linked to a solid substrate; wherein the solid substrate comprises a substrate surface and an association surface; wherein the association surface comprises a composition having a pI in a range of between about pI 5 to about pI 10, (c) contacting the test sample of step (a) with the association device of step (b) under conditions that induce the substrate surface or the association surface to have a net positive (cationic) charge density; (d) removing the test sample nucleic acid not associated to the probe of step (b) by washing; and, (e) detecting nucleic acid remaining associated to the nucleic acid probe after step (d).

As noted above, the device of the invention can be designed such that the net positive (cationic) charge density is close enough to the oligonucleotide probe to measurably influence the kinetics or specificity of association. Thus, the distance between the probe and the charged surface of the device can be about 100 angstroms, about 50 angstroms, about 30 angstroms, or about 20 angstroms, or, if appropriate, greater or lesser distances.

In other embodiments, the association conditions of further comprise no salt or low salt conditions. As noted above, low salt conditions comprise any set of conditions wherein the sodium ion (or equivalent ion) concentration is less than seen under physiologic conditions. Thus, low salt conditions can comprise, e.g., less than about 100 mM, about 75 mM, about 50 mM, about 25 mM, about 20 mM, about 15 mM, or about 5 mM, or about 2 mM sodium or equivalent cation. The salt can be sodium chloride, potassium chloride,

sodium phosphate, or equivalents. The salt can also include any alkaline metal, e.g., (in addition to K[redacted]s+, and equivalents, and other divalent cati[redacted].g., Mg++, and equivalents. Thus, in alternative embodiments, low salt conditions can comprise sodium phosphate or equivalent in these concentrations.

In various embodiments, the contacting of step (c) (e.g., the association step or hybridization step) is accomplished in no more than about 30 seconds; and, the wash step can be accomplished in no more than about 30 seconds. In an alternative embodiment, the contacting of step (c) and the washing step are both accomplished in no more than about 30 seconds.

In using the device of the invention and practicing the methods of the invention, conditions that induce the substrate surface or the association surface to have a net positive (cationic) charge density in the contacting of step (c) (e.g., the association step or hybridization step) comprise conditions having a pH below the pI of the composition (of the association surface). In another embodiment, the washing step further comprises conditions which alter the association surface (from a positive net charge density) to induce a neutral or net negative (anionic) charge density. These conditions can comprise a pH at or above the pI of the composition. For example, the composition can comprise streptavidin having a pI of about 5.5; and, the association step can be under conditions comprising a pH of below 5.5; and, the washing step can be under conditions above pH 5.5.

In another embodiment, the composition can comprise a primary amine group having a pI at about 10. The composition comprising the primary amine group can be selected from the group consisting of amino silane, polylysine, organic polyamines, or equivalents or mixtures thereof. When a primary amine comprises the association surface, the association can be under conditions comprising a pH of below 10. In alternative embodiments, the washing step can be under conditions above pH 10, or, the washing step can comprise use of a wash buffer comprising a composition that neutralizes or induces a negative (anionic) charge density on the association (e.g., hybridization) surface. The neutralizing composition in the wash step can comprise an anionic polymer, such as, e.g., oligo-aspartate, oligo-glutamate, polyvinyl sulfate, milk casein, or equivalent. The

neutralizing composition can also comprises a neutral polymer such as, e.g., one or a mixture of neutral polymers, including, e.g., polyvinyl pyrrolidone, ficol or BSA. As noted above, the association of the nucleic acid in the test sample to the nucleic acid probe can comprise Watson-Crick base pairing, Hoogstein based double or triple helix formation, reverse Hoogsteen hydrogen bonding interactions, and the like.

The invention also provides methods for detecting a single base pair difference between a nucleic acid in a test sample and an oligonucleotide probe comprising the following steps: (a) providing a test sample comprising a nucleic acid; (b) providing an association device comprising an oligonucleotide probe linked to a solid substrate; wherein the solid substrate comprises a substrate surface and an association surface; wherein the association surface comprises a composition having a pI in a range of between about pI 5 to about pI 10, wherein the distance between the oligonucleotide probe and the composition is no more than about 100 angstroms; (c) contacting the test sample of step (a) with the association device of step (b) under conditions that induce the substrate surface or the association surface to have a net positive (cationic) charge density under no salt or low salt conditions; (d) altering the association surface to an anionic environment by changing the conditions to comprise a pH that induces a net negative (anionic) charge density to the association surface, or, a neutral charge density by coating the association surface with a neutral or anionic polymer composition; (e) removing the test sample nucleic acid not associated to the probe of step (b) under the altered conditions of step (d); and (f) detecting nucleic acid remaining hybridized to the nucleic acid probe after step (e).

The invention also provides methods for detecting a single base pair difference between a nucleic acid in a test sample and an oligonucleotide probe comprising the following steps: (a) providing a test sample comprising a nucleic acid; (b) providing an association device comprising a plurality of immobilized oligonucleotide probes linked to a solid substrate comprising a polystyrene, wherein the solid substrate comprises a surface onto which a strepavidin has been immobilized, wherein the distance between the oligonucleotide probe and the positively charged surface is no more than about 100 angstroms; (c) contacting the test sample of step (a) with the association device of step (b) under no salt or

low salt conditions and a pH of below 5.5; (d) washing the oligonucleotide probes with a buffer comprising conditions over pH 5.5; (e) removing the test sample nucleic acid not specifically associated with the probe of step (b) under the wash conditions of step (d); and, (f) detecting nucleic acid remaining associated with the immobilized nucleic acid probes after step (e).

In the devices and methods of the invention, the oligonucleotides probes can be biotinylated and linked to the solid substrate by binding of the biotin to a high-affinity biotin binding ligand, e.g., strepavidin or avidin, anti-biotin antibody, or equivalents.

The invention also provides method for making a hybridization device comprising an oligonucleotide probe linked to a solid substrate comprising the following steps: (a) providing a clean polystyrene surface or equivalent; (b) providing a biotinylated oligonucleotide probe; (c) providing a strepavidin tetramer; (d) contacting the biotinylated oligonucleotide probe of step (b) with the strepavidin tetramer of step (c) in an aqueous solution; (e) applying the aqueous solution of step (d) directly to the polystyrene surface of step (a); and, (f) incubating the probe-applied polystyrene of step (e) in a humid environment for a sufficient amount of time to allow stable absorption of the strepavidin to the polystyrene surface. The ratio of biotinylated oligonucleotide probe to strepavidin tetramer in the solution can be about, e.g., four to one, about three to one, about two to one, or about one to one. The aqueous solution can comprise about 20 mM sodium acetate or equivalent at a pH of about 5.5. The application of solution can comprise depositing a plurality of solution microdeposits. In alternative embodiments, the microdeposits of solution can comprise, e.g., about 10 to about 50 nanoliters; or, a microdeposit solution fluid volume ranging between about 10 picoliters to about 100 nanoliters, between about 100 picoliters to about 50 nanoliter, or between about 1 nanoliter to about 20 nanoliters.

Association Devices comprising Porous Sold Substrates

The invention provides an porous association device comprising: a plurality of nucleic acid probes or polypeptide probes or a combination thereof linked to a solid substrate; wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end

opening to the external surface to allow passage of fluid into a pore, and wherein the pore surfaces comprise an association surface; wherein the association surface comprises a charged surface comprising net positive (cationic) charge density under conditions comprising a pH lower than the pI of the association surface; wherein the association surface comprises a charged surface comprising a net negative charge density under conditions comprising at a pH higher than the pI of the association surface; and, wherein the distance between the nucleic acid (e.g., oligonucleotide) probe or the polypeptide probe and the charged association surface is no more than about 100 angstroms.

In one embodiment, the association device further comprises an aqueous solution comprising a pH lower than the pI of the association surface, thereby inducing a net positive (cationic) charge density on the association surface. In devices with association surfaces induced to have a net positive charge, the net positive (cationic) charge density on the association surface can induce a net positive (cationic) charge density in the pore space of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of negatively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of positively charged molecules out of the pore space. In one embodiment, under conditions comprising a pH lower than the pI of the association surface, the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides into the pore space from the aqueous solution outside of the pores. Under conditions comprising a pH lower than the pI of the association surface, the net positive (cationic) charge density on the charged surface of the association surface can be at least about 10^{10} or at least about 10^{11} charges per square millimeter or a charge equivalent thereof.

In another embodiment, the porous association device of the invention comprises an aqueous solution comprising a pH higher than the pI of the association surface thereby inducing a net negative (anionic) charge density on the association surface. In one embodiment, under conditions comprising a pH higher than the pI of the association surface the net negative (anionic) charge density on the association surface induces a net negative (anionic) charge density in the pore space of the device, thereby generating a

thermodynamic partitioning equilibrium favorable to the movement of positively charged molecules into pore space from the aqueous solution outside the pores and favorable to the movement of negatively charged molecules out of the pore space. In one embodiment, under conditions comprising a pH higher than the pI of the association surface, the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides out of the pore space. In one embodiment, under conditions comprising a pH higher than the pI of the association surface, the net negative (anionic) charge density on the charged surface of association surface is at least about 10^{10} or at least about 10^{11} charges per square millimeter or a charge equivalent thereof.

In one embodiment, the porous association surface comprises a streptavidin linked to an oligonucleotide or a polypeptide probe. The streptavidin can be present on the association surface at a density of at least about 10^9 molecules, about 10^{10} molecules, or about 10^{11} molecules or about per square millimeter.

In one embodiment, the porous association surface comprises a histidine or histidine derivative linked to an oligonucleotide or a polypeptide probe. The histidine or histidine derivative can be present on the association surface at a density of at least about 10^9 molecules, about 10^{10} molecules, or about 10^{11} molecules per square millimeter.

In one embodiment, the porous association surface comprises an imidazole or an imidazole derivative linked to an oligonucleotide or a polypeptide. The imidazole or imidazole derivative can be present on the association surface at a density of at least about 10^9 molecules, about 10^{10} molecules, or about 10^{11} molecules per square millimeter.

In one embodiment, the porous association surface comprises a citrate or citrate derivative linked to an oligonucleotide or a polypeptide probe. The citrate or citrate derivative is present on the association surface at a density of at least about 10^9 molecules, about 10^{10} molecules, or about 10^{11} molecules per square millimeter.

In alternative embodiments, the internal pores comprise a diameter of at least about 10 angstroms to about 1000 angstroms in diameter, about 50 angstroms to about 700 angstroms, about 100 to about 600 angstroms, or about 500 angstroms.

In one embodiment, the porous solid substrate can form a side or a bottom of a well in a m[redacted]iter plate or an equivalent structure.

In alternative embodiments, the porous solid substrate comprises a porous bead or a porous microsphere or an equivalent structure; a porous polytetrafluoroethylene filter or a porous filter comprising an equivalent composition; a porous fiber, a porous hollow fiber or a porous fabric or an equivalent structure; a polyacrylamide or an equivalent polymer composition; a polymethacylamide, a methyl methacrylate, a glycidyl methacrylate, a dialkylaminoalkyl-(meth)acrylate, or a N,N-dialkylaminoalkyl (meth)acrylate, or an equivalent composition; an agarose or an equivalent polymer composition; a polyimid or an equivalent polymer composition; a controlled pore silica or porous glass or an equivalent composition; or, a porous foam or an equivalent composition. The porous foam can comprise a poly(D,L glycolic-co-lactic acid) or a poly(D, L-lactide-co-glycolide) (PLGA) or an equivalent composition. The porous solid substrate can also comprise a porous ceramic or an equivalent composition. The porous solid substrate can comprise a poly(ethylene glycol terephthalate) (PEGT) or a poly(butylene terephthalate) (PBT). The porous solid substrate can comprise a monodispersed carbon nanotube or a nanotube comprising an equivalent composition. The monodispersed carbon nanotube can comprise a patterned porous silicon or an equivalent composition. The porous solid substrate can comprise porous polystyrene or an equivalent composition. The polystyrene can comprise a porous poly(styrene-divinylbenzene) (PS-DVB) or an equivalent composition. The porous solid substrate can comprise a plastic or a plastic co-polymer or an equivalent thereof; a polyvinyl, a polypropylene or a polyester or an equivalent thereof; or, a poly(vinyl alcohol) (PVA) hydrogel nanoparticle.

In one embodiment, the porous solid substrate comprises a plurality of different nucleic acid (e.g., oligonucleotide) probes, wherein the probes are arranged in spatially defined areas over the surface or the pores or the fibers of the association device.

In one embodiment, the pores comprise a closed distal end. Alternatively, the pores can comprise an open distal end, wherein the open distal pore end allows passage of fluid through the pore. In one embodiment, substantially only the pore surface comprises an

association surface. In one embodiment, the distance between the nucleic acid (e.g., oligonucleotide probe and the charged surface is no more than about 100 angstroms, about 50 angstroms, about 30 angstroms, or about 20 angstroms. In one embodiment, the oligonucleotide or the polypeptide probe is at least about 11 to about 20 residues in length. In one embodiment, the nucleic acid or polypeptide probes are covalently attached to the association surface.

In one embodiment, the association surface comprises a streptavidin and the association surface has a net positive (cationic) charge density at a pH lower than pH 5.5 and the association surface has a net negative charge density at a pH higher than pH 5.5. In one embodiment, the association surface comprises a histidine or a histidine derivative and the association surface has a net positive (cationic) charge density at a pH lower than about pH 6.7 and the association surface has a net negative charge density at a pH higher than about pH 6.7. In one embodiment, the association surface comprises an imidazole or an imidazole derivative and the association surface has a net positive (cationic) charge density at a pH lower than about pH 6.0 and the association surface has a net negative charge density at a pH higher than about pH 6.0. In one embodiment, the association surface comprises citrate or citrate derivatives or a carboxylic acid. In one embodiment, the association surface comprises an amino acid or peptide linked to the solid substrate surface by its amino terminal end and an aminated oligonucleotide linked to the carboxy terminal end of the amino acid or peptide. The peptide can comprise $[(arg)_n\text{-pro}]_n\text{-arg}_n$, $[(arg)_n\text{-pro-gly}]_n\text{-arg}_n$, or $[(arg)_n\text{-gly-gly}]_n\text{-arg}_n$, wherein n is the integer 2, 3, 4, 5 or 6. The peptide can comprise $[(arg)_5\text{-pro}]_5\text{-arg}_5$, $[(arg)_5\text{-pro}]_4\text{-arg}_5$, $[(arg)_5\text{-pro-gly}]_3\text{-arg}_5$, $[(arg)_5\text{-pro-gly}]_4\text{-arg}_5$, $[(arg)_5\text{-gly-gly}]_3\text{-arg}_5$, or $[(arg)_5\text{-gly-gly}]4\text{-arg}_5$.

The invention provides a method for associating a nucleic acid or a polypeptide in a sample to a nucleic acid or a polypeptide probe comprising the following steps: (a) providing a test sample comprising an aqueous solution comprising a nucleic acid or a polypeptide or a combination thereof, (b) providing a porous association device of the invention; and, (c) contacting the test sample of step (a) with the association device of step (b) under contacting conditions comprising a pH higher or lower than the pI of the

associati[redacted]rface, thereby inducing a net positive or net[redacted]tive charge density on the association surface. In one embodiment, the contacting conditions comprise a pH higher or lower than the pI of the association surface, wherein these conditions are established before contacting the test sample of step (a) with the association device of step (b). In one embodiment, a desired pH is established by contacting the device with an aqueous solution buffered to a desired pH. In one embodiment, where the contacting conditions comprise a pH lower than the pI of the association device, sufficient net positive charge density is induced on the association surface to generate a net positive charge density in the pore of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of negatively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of positively charged molecules out of the pore space. In one embodiment, where the contacting conditions comprise a pH lower than the pI of the association device, the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides into the pore space from the aqueous solution outside of the pores.

In an alternative embodiment, where the contacting conditions comprise a pH higher than the pI of the association device, sufficient net negative charge density is induced on the association surface to generate a net negative charge density in the pore of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of positively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of negatively charged molecules out of the pore space. In one embodiment, where the contacting conditions comprise a pH higher than the pI of the association device, the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides outside of the pores.

In another embodiment, the method further comprises removing a nucleic acid or a polypeptide not associated with a probe by washing with a buffered aqueous solution. The wash conditions can induce or maintain a net positive charge density on the surface of the device if the washing (contacting) step comprises net positive charge density conditions,

or, wherein the wash conditions induce or maintain a net negative charge density on the surface of the device if the wash (contacting) step comprises net negative charge density conditions. The method can further comprise detecting the nucleic acid or polypeptide remaining associated with the nucleic acid or polypeptide probe after the washing and removal of non-associated sample nucleic acid or polypeptide. In one embodiment, the method further comprises removing the nucleic acid or polypeptide by washing under conditions comprising a pH higher than the pI of the association surface if the contacting conditions step comprises a pH lower than the pI of the association surface, or, by washing in an aqueous solution comprising a pH lower than pI of the association surface if the contacting conditions step comprises a pH higher than the pI of the association surface.

The invention also provides a method for detecting at least a single base pair difference between a nucleic acid in a test sample and an oligonucleotide probe comprising the following steps: (a) providing a test sample comprising a nucleic acid; (b) providing a porous association device of the invention; (c) contacting the test sample with the porous association device under conditions that induce the substrate surface or the association surface to have a net positive (cationic) charge density under no salt or low salt conditions; (d) altering the association surface to an anionic environment by changing the conditions to comprise a pH that induces a net negative (anionic) charge density to the association surface, or, a neutral charge density by coating the association surface with a neutral or anionic polymer composition; (e) removing test sample nucleic acid not associated with a probe of the device of step (b) under the altered conditions of step (d); and, (f) detecting nucleic acid remaining hybridized to the nucleic acid probe after step (e).

The invention also provides a method for making a hybridization device comprising a nucleic acid (e.g., oligonucleotide) probe linked to a solid substrate comprising the following steps: (a) providing a clean porous polystyrene surface or equivalent; (b) providing a biotinylated oligonucleotide probe; (c) providing a strepavidin tetramer; (d) contacting the biotinylated oligonucleotide probe of step (b) with the strepavidin tetramer of step (c) in an aqueous solution; (e) applying the aqueous solution of step (d) directly to the polystyrene surface of step (a); and, (f) incubating the probe-applied polystyrene of step (e)

in a humi[redacted]ironment for a sufficient amount of time to [redacted]v stable absorption of the strepavidin to the polystyrene surface.

The invention also provides a method for making a porous surface of an association device comprising an oligonucleotide or polypeptide probe linked to a solid substrate, wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end opening to the external surface to allow passage of fluid into the pores, and wherein the pore surfaces comprise an association surface, comprising: (a) co-polymerizing streptaviden and biotinylated nucleic acid probes into a mixture of acrylamide and bisacrylamide, under conditions wherein a porous matrix polymerizes; or, (b) co-polymerizing streptaviden into a mixture of acrylamide and bisacrylamide, under conditions wherein a porous matrix polymerizes, and, after polymerization, adding biotinylated nucleic acid probe to the polymerized porous matrix by perfusion. In one embodiment, the mixture in step (a) or step (b) comprises about 19% acrylamide and about 1% bisacrylamide, or amounts giving equivalent results. In one embodiment, the final concentration of streptaviden in the mixture of step (a) or step (b) is about 10^{-6} M streptaviden tetramer.

The invention also provides a method for making a porous surface of an association device comprising an oligonucleotide or polypeptide probe linked to a solid substrate, wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end opening to the external surface to allow passage of fluid into the pores, and wherein the pore surfaces comprise an association surface, comprising the following steps: (a) providing a porous silica matrix; (b) providing a solution of activated silane; (c) contacting the porous silica matrix of step (a) with the activated silane of step (b) by a gas phase or fluid phase deposition.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention, which are given for the purpose of disclosure, when taken in conjunction with the accompanying drawings.

All publications, patents and patent applications cited herein are hereby expressly incorporated by reference for all purposes.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation showing modulation of duplex formation by surface physical chemistry.

Figures 2A, 2B and 2C are schematic representations of probe coupling methodologies.

Figure 3 shows the surface state ionic strength dependence of K-ras 12 mer duplexes.

Figures 4A and 4B demonstrate the Ph effects on the electrostatic nature of a prototypical "smart" surface (streptavidin) as revealed by dissociation kinetics of hybridization at pH 7.2 or pH 5.2.

Figures 5A and 5B demonstrate the Ph and cation effects on dissociation kinetics.

Figure 6 is a schematic of a secondary structure assay.

Figures 7A and 7B show hybridization of a hairpin forming target at low ionic strength. Signal intensity given in Relative Light Units (RLUs) from a luminometer.

Figure 8 is a schematic of duplex formation in a protamine model.

Figures 9A and 9B show selectivity enhancement by Salmine A1 in Na^+ solutions (Fig. 9A) versus protamine solutions (Fig. 9B).

Figure 10 shows the results of hybridization on the surface with a 19 mer oligonucleotide.

Figures 11A and 11B are schematic representations of an amino acid hybridization surface showing a plurality of amino acids.

Figures 12 to 17 are schematic representations of various hybridization surfaces on solid substrates.

Figure 18 illustrates data generated by experiments discussed in Example 13, below. Figure 18A shows data demonstrating the slow kinetics of a k-ras 1 amplicon hybridization to probes immobilized on a streptavidin surface at high salt and high pH.

Solid: pe[redacted]match (k1). Open: single G-T mismatch (k3[redacted]e binding). All other mismatches gave hybridization signals which were indistinguishable from background. Figure 18B shows data demonstrating the fast kinetics of k-ras amplicon hybridization to the probes immobilized on a streptavidin surface at low salt and low pH. Solid: perfect match (k1). Open: single G-T mismatch (k3 probe binding).

The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness. It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

Detailed Description

The invention provides devices and methods for detecting nucleic acids in samples using immobilized probes. The devices and methods of the invention provide a nucleic acid association (including, e.g., "hybridization") enhancement effect and high sequence selectivity. As discussed below, the observed association-hybridization enhancement is a general property of the interaction between DNA and immobilized probes on the invention's charge-adjustable, or "tunable" device surfaces.

The invention is not limited by what molecular, physical or kinetic mechanisms are underlying the observed effects. However, the data presented here demonstrated that the invention successfully exploits the physical chemistry of device surfaces for the purpose of DNA association-hybridization rate enhancement. In particular, the data demonstrated that very significant enhancement of hybridization rates can be obtained at room temperature, while retaining high selectivity, using the DNA microarray devices of the invention. In the methods and devices of the invention, the applied device surface effects give rise to hybridization under solution state conditions of low salt (e.g., from 0 to about 2 mM) and room temperature (e.g., about 25°C) which are denaturing with respect to undesired secondary structure within target (test sample nucleic acid) strands. Thus, the invention provides minimization of the effect of target structure as a side reaction to high precision DNA microarray applications.

e "tunable surface" association devices (e.g. DNA microarray devices) of the invention can be mass-produced at low cost. Use of the methods and devices of the invention provides the ability to drive hybridization of test sample nucleic acid to immobilized probe to completion in a few seconds. This very significant increase in rate is of practical significance in the population scale application of DNA microarrays, such as, e.g., for use in pharmacogenomics, where hundreds or thousands of samples are to be processed in parallel, or for the use of DNA microarrays in point-of-care applications which would require a very rapid data report on single samples.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "a charge" as used herein means the equivalent of one positive (cationic) or one negative (anionic) charge; e.g., the equivalent of the amount of charge a strepavidin molecule (or other "tunable" molecule used in the association devices of the invention) under conditions comprising a pH lower than the pI of strepavidin has (i.e., a single positive charge), or, when the pI is higher than the pI of strepavidin, the negative charge. For example, in one embodiment, a tunable surface of a device of the invention comprises at least about 10^{11} charges per square millimeter.

The term "nucleic acid" as used herein refers to a deoxyribonucleotide or ribonucleotide in either single- or double-stranded form. The term encompasses nucleic acids, i.e., oligonucleotides, containing known analogues of natural nucleotides which have similar or improved binding properties, for the purposes desired, as the reference nucleic acid. The term also encompasses nucleic-acid-like structures with synthetic backbones. DNA backbone analogues provided by the invention include phosphodiester, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, morpholino carbamate, and peptide nucleic acids (PNAs); see Oligonucleotides and

Analogues: A Practical Approach, edited by F. Eckstein, IRL Press at Oxford University Press (1991); Antisense Strategies, Annals of the New York Academy of Sciences, Volume 600, Eds. Baserga and Denhardt (NYAS 1992); Milligan (1993) J. Med. Chem. 36:1923-1937; Antisense Research and Applications (1993, CRC Press). PNAs contain non-ionic backbones, such as N-(2-aminoethyl) glycine units, and can be used as probes (see, e.g., U.S. Patent No. 5,871,902). Phosphorothioate linkages are described in WO 97/03211; WO 96/39154; Mata (1997) Toxicol. Appl. Pharmacol. 144:189-197. Other synthetic backbones encompasses by the term include methyl-phosphonate linkages or alternating methylphosphonate and phosphodiester linkages (Strauss-Soukup (1997) Biochemistry 36:8692-8698), and benzylphosphonate linkages (Samstag (1996) Antisense Nucleic Acid Drug Dev 6:153-156). Modified internucleoside linkages which are resistant to nucleases are described by, e.g., U.S. Patent No. 5,817,781. The term nucleic acid is used interchangeably with the terms gene, cDNA, mRNA, oligonucleotide primer, probe and amplification product.

The term "oligonucleotide probe" as used herein defines a molecule comprised of more than three deoxyribonucleotides or ribonucleotides. The exact length will depend on many factors leading to the ultimate function or use of the oligonucleotide probe, including temperature, source of the probe and use of the method. The oligonucleotide probe can occur naturally as in a purified restriction digest or be produced synthetically. The oligonucleotide probe is capable of binding to any nucleic acid (e.g., DNA or RNA) target when placed under conditions which induce binding of the target to the oligonucleotide probe. In the device and methods of the present invention, the oligonucleotide probes can be at least greater than about 10 mer in length, about 20-mer in length, or can range from about 10 to 30 to 50 mer, or greater. Sensitivity and specificity of the oligonucleotide probes are determined by the probe length, uniqueness of sequence and localized environment. Probes which are too short, for example less than 10 mer, may show non-specific binding to a wide variety of sequences in the DNA or RNA. The probes of the invention can be linear or circular or stem-loop. For example, single-stranded circular oligonucleotides can bind to both single-stranded and double-

stranded targ[redacted]cleic acids by Watson-Crick base pairing or [redacted]gstein associations, as discussed below (see, e.g., U.S. Patent Nos. 5,683,874; 5,674,683; 5,514,546.

It is known that probes which are substantially complementary to a strand of DNA or RNA will bind to that specific strand of DNA and RNA. Thus, in order for a probe to bind, the probe sequence need not reflect the exact complementary sequence of the DNA or RNA, however, the more closely it does reflect the exact sequence the better the binding. This ability to bind without the exact sequences reflects the fact that the probe can bind the DNA or RNA where there is a "base mismatch". The term "base mismatch" refers to a change in the oligonucleotide such that when the probe lines up with the known sequence an abnormal bonding pair of nucleotides is formed. Normally guanine (G) and cytosine (C) bind and adenine (A) and thymine (T) bind in the formation of double-stranded nucleic acids. Thus, the standard base pairing, A-T or G-C is not seen in base mismatch pairing. A variety of base mismatches can occur, for example G-G, C-C, A-A, T-T, A-G, A-C, T-G or T-C. This mispairing and the effects of localized environments on the efficiency of binding is used in the present invention to detect the mispairing. When there are base mismatches between a probe and DNA or RNA, the probe will bind preferentially to the strand that has the fewest base mismatches under the most stringent conditions. The method of the present invention provides a way to alter the conditions such that the combination with the fewest base mismatches will preferentially bind to the probe.

As used in the present invention a "solid substrate" or "substrate surface" is the material which forms the solid support for the device or the association (e.g., hybridization) reaction. It is composed of a substrate surface and an association (e.g., hybridization) surface. The substrate surface can be selected from a variety of materials including, e.g., polyvinyl, polystyrene, polypropylene, polyester, other plastics, glass, SiO_2, other silanes, gold or platinum, see further examples described, below. The solid surfaces can be derivatized, e.g., thiol-derivatized biopolymers (including nucleic acid or oligonucleotide probes) and organic thiols can be bound to a metal solid substrate; see, e.g., U.S. Patent No. 5,942,397 (see below for more examples).

In some embodiments of the invention, bet[redacted] the surface of the solid substrate and the oligonucleotide probe, is an "association" surface. The association surface comprises a composition that binds to the solid surface, either covalently or non-covalently. The oligonucleotide probe is immobilized to the device by binding (covalently or non-covalently) to the attached composition. As discussed above, the device of the invention promotes the association of nucleic acid in a sample applied to the device to the immobilized oligonucleotide. This can include, e.g., increased kinetics of association or disassociation (which, in some embodiments, can depend on whether the conditions of association comprise a net cationic charge or a net anionic charge, respectively).

The term "association" includes, e.g., hybridization (e.g., Watson-Crick base pairing), Hoogsteen-based double or triple helix formation, reverse Hoogsteen hydrogen bonding interactions, and the like. See, e.g., Giovannangeli (1997) Antisense Nucleic Acid Drug Dev. 7:413-421; U.S. Patent Nos. 5,473,060; 6,004,750. Hoogsteen triple-helical three-way junctions (ternary complexes) can be designed from the same sequences used for the Watson-Crick triple-helical three-way junctions, see, e.g., Husler (1994) Arch. Biochem. Biophys. 313:29-38; Husler (1995) Arch. Biochem. Biophys. 322:149-166. For example, the invention can comprise oligonucleotides (also called foldback triplex-forming oligonucleotides) that can hybridize to single-stranded complementary polypurine nucleic acid targets by Hoogsteen base pairing as well as by Watson-Crick base pairing (see, e.g., Kandimalla (1995) Nucleic Acids Res. 23:1068-74). Nucleic acid analogs can also be used to design the oligonucleotide probes of the invention, e.g., peptide nucleic acids (PNAs) and analogues of peptide nucleic acids can be designed to form duplex, triplex, and other structures with nucleic acids, see, e.g., U.S. Patent No. 5,986,053. Thus, the invention can also be used to isolate or detect double stranded nucleic acids without the need for denaturing before application to the device; e.g., double-stranded DNA complexes to the immobilized oligonucleotide probe to generate a triple helix. The DNA of the sample can then be washed off, or recovered intact, by treating (washing) with a reagent that breaks the bonds between the oligonucleotide and the intact double stranded DNA while not affecting the Watson-Crick base pairs of the double helix. See, e.g., U.S. Patent No. 5,482,836.

one embodiment of the methods of the inve , the test sample nucleic acid not specifically associated with the immobilized probe is removed. The term "not specifically associated" means that the association, or binding, of the probe to the test sample nucleic acid is of relatively low, or "non-specific" affinity; e.g., sample nucleic acid not "specifically associated" with probe can be washed off under conditions equivalent to those used in as wash conditions in the methods of the invention, e.g., wherein the surface of the device has a neutral or negative net charge density and the wash conditions comprise low salt conditions (see definition below).

The term "immobilized" as with "immobilized nucleic acid probe" means that the oligonucleotide probe can be attached to a surface (e.g., the solid support surface or an association surface of the device of the invention) in any manner by and methods; including, e.g., reversible or non-reversible binding, covalent or non-covalent attachment, and the like.

The term "low salt conditions" includes any set of conditions wherein the sodium or equivalent cation concentration is below that of physiologic conditions; for example, in various embodiments, low salt conditions comprise about 100 mM, about 75 mM, about 50 mM, about 25 mM, about 20 mM, about 15 mM, or about 5 mM, or about 2 mM, or less than 2 mM sodium or equivalent cation (such conditions include, e.g., sodium phosphate, sodium chloride, and the like).

The term "streptavidin" as used herein includes all derivatives and analogs that are functional equivalents, particularly the ability to bind biotin or equivalents thereof and have a pI within an upper an lower range compatible to use of the "tunable" surface of the invention, as described herein, e.g., as neutraviden (although streptavidin has a pI of 5.5, streptavidin derivatives and analogs are not limited to such a pI). Streptavidin derivatives and analogs are well known in the art and are described, e.g., in 5,973,124; 5,846,537; 5,672,691; 5,489,528; 5,328,985; 5,272,254; Stayton (1999) Biomol. Eng. 16:93-99; Webber (1989) Science 243:85-88; Wilchek (1988) Analytical Biochem. 171:1-32. Bayer (1990) J. of Chromatography 510:3-11; which can also be used to design equivalents and derivatives for use on "tunable surfaces" of the invention.

Nucleic A[redacted]and Oligonucleotide Probes

This invention provides devices and methods for use in the detection and/or isolation of nucleic acids. The devices and methods of the invention use immobilized oligonucleotide probes. These probes can be made and expressed *in vitro* or *in vivo*, any means of making and expressing nucleic acids and probes used in the devices or practiced with the methods of the invention can be used. The invention can be practiced in conjunction with any method or protocol known in the art, which are well described in the scientific and patent literature.

The nucleic acid sequences of the invention, e.g., probes of the devices, whether, e.g., RNA, cDNA, fragments of genomic DNA, can be isolated from a variety of sources, genetically engineered, amplified, and/or expressed recombinantly. Any recombinant expression system can be used, including, in addition to mammalian cells, e.g., bacterial, yeast, insect or plant systems.

Alternatively, these nucleic acids can be synthesized *in vitro* by well-known chemical synthesis techniques, as described in, e.g., Carruthers (1982) Cold Spring Harbor Symp. Quant. Biol. 47:411-418; Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066. Double stranded DNA fragments may then be obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence.

Techniques for the manipulation of nucleic acids, such as, e.g., generating mutations in sequences, subcloning, labeling probes, sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND

MOLECULAR [redacted]OGY: HYBRIDIZATION WITH NUCLEIC ACID [redacted]ES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

Nucleic acids can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, e.g., analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, e.g. fluid or gel precipitin reactions, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis (e.g., SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Oligonucleotide primers can be used to generated device probes or to amplify an "associated" or hybridized nucleic acid for detection or replication purposes. These techniques can also be used for site-directed mutagenesis (e.g., to generate alternative probes). Amplification methods are also well known in the art, and include, *e.g.*, polymerase chain reaction, PCR (PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS, ed. Innis, Academic Press, N.Y. (1990) and PCR STRATEGIES (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) Genomics 4:560; Landegren (1988) Science 241:1077; Barringer (1990) Gene 89:117); transcription amplification (see, e.g., Kwoh (1989) Proc. Natl. Acad. Sci. USA 86:1173); and, self-sustained sequence replication (see, e.g., Guatelli (1990) Proc. Natl. Acad. Sci. USA 87:1874); Q Beta replicase amplification (see, e.g., Smith (1997) J. Clin. Microbiol. 35:1477-1491), automated Q-beta replicase amplification assay (see, e.g., Burg (1996) Mol. Cell. Probes 10:257-271) and other RNA polymerase mediated techniques (*e.g.*, NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) Methods Enzymol. 152:307-316; Sambrook; Ausubel; U.S. Patent Nos. 4,683,195 and 4,683,202; Sooknanan (1995) Biotechnology 13:563-564.

Associa[redacted] Hybridizati n Devices

Association/ Hybridization Surface Compositions

In the device of the invention, the solid support surface comprises an "association" surface, which, e.g., can be a "hybridization" surface. The association/ hybridization surface can be selected from a variety of materials, e.g., including those shown in Table 1 (Example 11) and other materials such as organic acids, inorganic acids, organic bases and inorganic bases. This can include amino acids, peptides, oligopeptide, polypeptides, peptidomimetics, other short polymers or organic molecules. When amino acids are used, alternative embodiment can use methyl esters because of commercial availability and the fact that they are not altered by the formation reactions (binding of the association surface to the support surface).

"Peptidomimetics" include synthetic chemical compounds that have substantially the same structural and/or functional characteristics of the corresponding composition, e.g., the peptides, oligopeptides (e.g., oligo-histidine, oligo-aspartate, oligo-glutamate, poly-$(his)_2(gly)_1$, and poly-$(his)_2(asp)_1$), polypeptides, imidazole derivatives or equivalents used in the association surface of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity. Individual peptidomimetic residues can be joined by peptide bonds, other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropyl-carbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., $-C(=O)-CH_2-$ for $-C(=O)-NH-$), aminomethylene (CH_2-NH), ethylene, olefin (CH=CH), ether (CH_2-O), thioether (CH_2-S), tetrazole (CN_4-), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, Marcell Dekker, NY).

e "association" surface, or hybridization su[redacted], can also be an "effective association surface" (e.g., "effective hybridization surface"). For example, an oligonucleotide probe (e.g., modified probes) can have a sufficient charge that when linked to the solid substrate, it creates a localized positive, neutral or negative environment at the surface of the solid substrate. This localized environment contributes to creation of the hybridization surface.

Accordingly, the support surface (comprising an "association" surface or an "effective association surface") can provide positive, neutral or negative charge density environments used to practice the present invention. Significantly, the device of the invention can provide different charge density conditions; and, in alternative embodiments, the charge density conditions of the surface of the device can be changed (i.e., the device is "tunable" to different electrostatic/ charge densities, including cationic, neutral or anionic conditions). Not all of the device need be the same charge density; for example if an exemplary device comprises a plurality of wells to provide a plurality of association/ hybridization microenvironments, each well can have a different charge density. Similarly, alternative devices of the invention can have a plurality of different solid support surfaces, association surfaces, or both.

The association / hybridization surface can be composed of a single compound or be composed of a combination of (e.g., combinatorial association) or a plurality of different compounds. One skilled in the art, using the teachings of, e.g., the combinatorial method herein, will be readily able to determine, without undue experimentation, the association / hybridization surface compounds or plurality of compounds which are most efficient for a desired set of conditions, e.g., a given oligonucleotide probe (e.g., its length, source, G/C content), how the probe has been modified (e.g., conjugated to biotin), for the probe to bind to the association / hybridization surface, for, e.g., distinguishing specific DNA or RNA target sites, specific Watson-Crick base pairing or Hoogstein base associations, isolating nucleic acid from sample, and the like.

The physical outcome of association / hybridization performed on a surface can include the association / hybridization surface itself as an integral part of the binding

reaction. [redacted]s is illustrated in Figure 1. An important as[redacted]f this illustration is the availability of free reactive groups on the surface. These free reactive groups can be secondarily modified after the probe linkage to the association / hybridization surface. This secondary modification can be performed with an extremely large repertoire of monomeric or oligomeric molecules using the same straightforward coupling chemistry used to link the probes. Examples of such molecules include all primary and modified amino acids, oligopeptides, polysaccharides, lipids and at least tens of thousands of other small organic molecules. The association / hybridization surface can include either only one of these molecules on the surface or some combination of these molecules. Thus, at least one molecule is used to alter the surface charge and water molecule binding (wetting) of the association / hybridization surface.

One skilled in the art of course recognizes that the ability to evaluate such a large library of potentially beneficial surface modifications with regard to association / hybridization rate enhancement or specificity is highly dependent on the availability of high-throughput screening methodologies. In one embodiment incorporating high-throughput uses, robotically placed probe arrays are placed on the surface of microtiter plate wells. Using this technology, each microtiter well of a 96-well plate can contain a probe array representing a desired combination of matched and mismatched probes with regard to target specificity. Additionally, each well can contain some variant or surface modification or can be subjected to different bulk solution components. Thus, one 96-well microtiter plate in which each well contains 16 probes can yield, e.g., 16 x 96 = 1,536 individual hybridization data points.

One embodiment of the present invention is an association/ hybridization device which exploits surface physical chemistry to enhance the selectivity and sensitivity of association/ hybridization assays for detecting DNA or RNA sequences. This association/ hybridization device comprises a solid substrate and an oligonucleotide probe, wherein the solid substrate includes an association/ hybridization surface having a neutral or negative charge density, said association/ hybridization surface accessible for linking to the oligonucleotide probe by a covalent linkage or a slowly reversible, non-covalent linkage.

e oligonucleotide probes of the device of th ention are linked to the association/ hybridization surface at a distance within which the charge density of the association surface (or effective association surface) or solid support surface can measurably influence the specificity or kinetics of probe-nucleic acid (of test sample) association and/or dissociation; typically, this is no more than about 100 angstroms. However, one skilled in the art will recognize that this distance is not an exact measurement. The magnitude of the surface effect (on, e.g., base pairing, Hoogstein associations, and the like) decreases progressively as the distance of the association event from the net surface charge increases. When the distance is about 100 angstroms, the net charge "surface effect" usually begins to become negligible relative to the bulk solution effect; however, under some circumstances the distance can be greater than 100 angstroms and remain significant. For example, the distance (between net electrostatic charge density of the device and the nucleic acid to interact with the immobilized probe) is selected to allow for the desired amount of physical or electrostatic interaction between the target nucleic acid (e.g., DNA or RNA) and the support surface or hybridization surface. The distance effects the binding equilibrium. The physical or electrostatic interaction can include, e.g., an interaction between the duplex and (i) surface electric field, or (ii) the local high cation concentration near the surface.

In specific embodiments, the probe is linked at a distance of no more than about 75 angstroms, about 50 angstroms, about 40 angstroms, or about 30 angstroms from the association/ hybridization surface, or, the linkage is about 20 angstroms.

In other specific embodiments, the support surface can be made negatively charged by layering a thin negatively charged protein film on the solid substrate.

Solid substrate surfaces

The invention provides associate/ hybridization devices comprising an oligonucleotide probe linked to a solid substrate. The surface can be of a rigid, semi-rigid or flexible material. The surface can be flat or planar, be shaped as wells, raised regions, etched trenches, pores, beads, filaments, or the like. Solid substrates can be of any material upon which an oligonucleotide can be directly or indirectly bound, or any surface upon which an association surface can be directly or indirectly bound. For example, suitable materials can

include p[redacted] glass (see, e.g., U.S. Patent No. 5,843,767) [redacted]mics, quartz or other crystalline substrates (e.g. gallium arsenide), metals, metalloids, polacryloylmorpholide, various plastics and plastic copolymers, Nylon™, Teflon™, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polystyrene/latex, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF) (see, e.g., U.S. Patent No. 6,024,872), silicones (see, e.g., U.S. Patent No. 6,096,817), polyformaldehyde (see, e.g., U.S. Patent Nos. 4,355,153; 4,652,613), cellulose (see, e.g., U.S. Patent No. 5,068,269), cellulose acetate (see, e.g., U.S. Patent No. 6,048,457), nitrocellulose, various membranes and gels (e.g., silica aerogels, see, e.g., U.S. Patent No. 5,795,557), paramagnetic or superparamagnetic microparticles (see, e.g., U.S. Patent No. 5,939,261) and the like. The surface can be derivatized for application of the association surface or other compounds. Reactive functional groups can be, e.g., hydroxyl, carboxyl, amino groups or the like. Silane (e.g., mono- and dihydroxyalkylsilanes, aminoalkyltrialkoxysilanes, 3-aminopropyltriethoxysilane, 3-aminopropyltrimethoxysilane) can provide a hydroxyl functional group for reaction with an amine functional group.

In one specific embodiment, the solid substrate is polystyrene, the association/ hybridization surface is streptavidin, and the oligonucleotide probe has been modified to include biotin and the modified probe is linked to the association/ hybridization surface by a non-covalent interaction of the biotin with the streptavidin.

In another specific embodiment the solid substrate is polyvinyl, polystyrene, polypropylene or polyester, the association/ hybridization surface is a thin carboxylic acid surface placed on the solid substrate and the oligonucleotide probe has been modified to include an amine group and the modified probe is covalently linked to the association/ hybridization surface.

In another specific embodiment, the oligonucleotide probe is linked to a glass solid substrate by an epoxysilane linkage to a terminal amine modification. This linked solid substrate/probe creates an effective association/ hybridization surface because the probe is sufficiently negative to provide a negatively charged environment in the probe/solid substrate localized area.

another specific embodiment, the associati bridization surface has a composition which switches electrostatic charge in the range of pH 5 to 8.

In a specific embodiment, the association/ hybridization device has a association/ hybridization surface where the electrostatic field is maintained at a negative potential. This allows for field-induced destabilization of mismatched binding.

In another embodiment the association/ hybridization surface has a composition where the surface electrostatic field is more cationic during association/ hybridization and serves as a nucleic acid attractor, but becomes more anionic during washing so as to create a field-induced destabilization of association or mismatch binding during washing.

One embodiment of the present invention is the use of film chemistries to produce an association/ hybridization surface which has switchable ("tunable") electrostatics. For example, in one embodiment, the surface is cationic or neutral at a pH of about 5 to 6 so as to attract nucleic acid targets to the surface during hybridization, but which become negatively charged at a pH of about 7 to 8 to confer a selective electrostatic interaction between target molecules and the surface during washing. As discussed herein, the net electrostatic charge density of a device of the invention is dependent on the pI of the composition (or net pI, if more than one composition) comprising the association surface and/or solid support surface.

In one specific embodiment of the charge "switching" ("tuning") effect, the composition of the association/ hybridization surface is selected from the group consisting of streptavidin, imidazole derivatives, carboxylic acids and other groups with pK values near neutrality. This can include histidine derivatives and citrate. It should also be noted that although an individual carboxylic acid or imidazole may not have a pK near neutrality the pK may be effectively, near neutral when various combinations of compounds are used. In another embodiment the association / hybridization surface can comprise arginine or arginine derivatives. Further, the skilled artisan will recognize that the association/ hybridization surface can include, e.g., amino acids, amino acid esters or a mixture of thereof.

A specific embodiment includes a associat[redacted]hybridization surface comprised of a Salmine A1-probe chimera linked to the support surface.

Another specific embodiment includes a association/ hybridization surface comprising a Salmine A1 and or Salmine A1-probe chimera. This association/ hybridization surface is selected to create highly selective association/ binding of target to the surface in the absence of exogenous salt ions.

Porous solid substrate surfaces

The invention provides associate/ hybridization devices comprising an oligonucleotide probe linked to a solid substrate, including semi-rigid or flexible materials that can be shaped as wells, raised regions, etched trenches; including porous beads, porous particles, porous membranes, microchanneled devices, nanotubes, filaments, or mixtures thereof or equivalents thereof. Any porous solid surface or method of making such a porous surface can be used to make and use the invention. For example, U.S. Patent No. 5,695,640 describes a hydrophobic porous substrate having coated on at least one external surface and on the internal surface a polyamide. Porous membranes are described, e.g., in Kwon (2000) J. Biomed. Mater. Res. 50:82-89. Porous foams of macroporous hydrogels are described, e.g., in Courtney (1999) J. Biomater. Sci. Polym. Ed. 10:1063-1077. Low density open cell organic foams are described, e.g., in U.S. Patent No. 5,945,084. Amorphous poly(D,L) glycolic-co-lactic acids are described, e.g., in Ranucci (1999) Tissue Eng. 5:407-420. Porous ceramics are described, e.g., in Ohgushi (1999) J. Biomed. Mater. Res. 48:913-927; and, ceramic porous membranes are described, e.g., in U.S. Patent Nos. 6,077,800; 5,269,926. Porous sol-gels are described, e.g., in Juszczak (1999) J. Biol. Chem. 274:30357-30360. Porous polytetrafluoroethylene filters are described, e.g., in Holmquist (1999) J. Biochem. Biophys. Methods 41:49-60. Porous matrices of poly(ethylene glycol terephthalate) (PEGT) and poly(butylene terephthalate) (PBT) are described, e.g., in van Dorp (1999) J. Biomed. Mater. Res. 47:292-300. Monodispersed carbon nanotubes are described, e.g., in Fan (1999) Science 283:512-514. Porous glycidyl methacrylate (GMA-GDMA) beads are described, e.g., in Malmsten (1999) J. Colloid Interface Sci. 220:436-442. Porous poly(styrene-divinylbenzene) (PS-DVB) particles are described, e.g., in Leonard (1999) J. Colloid

Interface Sci[redacted]:380-386. Porous polyacrylonitrile beads a[redacted]cribed, e.g., U.S. Patent No. 5,047,437. A hydrophilic porous membrane of a non-crystalline hydrophobic polymer and an in situ crosslinked hydrophilic acrylate polymer is described, e.g., in, U.S. Patent No. 6,083,393. Surface-grafted microporous membranes are described, e.g., in U.S. Patent No. 5,547,575. Microporous polyvinylidene fluoride membranes are described, e.g., in U.S. Patent No. 5,531,900. Porous membranes comprising cellulose compounds are described, e.g., in U.S. Patent No. 5,980,746. A microporous membrane comprising a partially fluorinated polyolefin membrane is described, e.g., in, U.S. Patent No. 5,209,849. A microfabricated thin-film membrane filter is described, e.g., in, U.S. Patent No. 5,985,328.

Various forms of porous beads, membrane, particles, nanotubes and microchannel devices and the like, are also described in, e.g., U.S. Patent Nos. 5,106,502; 5,022,999; 5,228,994; 5,847,075; 5,871,722; 5,889,073; 5,975,426; 6,007,690; WO 99/19717; which can be also be used to design alternative forms of porous solid surfaces for use in the invention.

In one embodiment, a porous solid substrate is made by polymerization of acrylamide-bisacrylamide. "Tunable elements" (e.g., streptavidin, histidine, imidazole, peptides, citrate or citrate derivatives or a carboxylic acid, and the like, as described herein) are introduced into the porous phase either by entrapment in the pores during the process of manufacture of the porous substrate matrix (e.g., as in the polymerization of acrylamide and the entrapment of streptaviden tetramer or peptides) or by direct covalent linkage of tunable elements. For example, in one embodiment, the method comprises co-polymerizing streptaviden into a mixture of about 19% acrylamide and about 1% bisacrylamide.

Covalent linkage can be obtained by linkage of the amino terminus of the peptide or the lysine side chains of streptaviden to a carboxylate or succimidate or by an NHS ester moiety, which can introduced into the porous polymer matrix.

In another embodiment, the method involves the use of a porous silica matrix. Tunable elements can be linked by coating the pores of the matrix with a monolayer of an activated silane (such as an epoxysilane) via gas phase or fluid phase deposition. The tunable elements are introduced by covalent linkage to the silane layer as through covalent coupling

to the terminal amine group of a peptide, or by covalent attachment of streptaviden or a streptaviden derivative, such as neutraviden. In one embodiment, the method comprises co-polymerization of streptaviden into an about 19% acrylamide, 1% bisacrylamide matrix at the time of polymerization. The concentration of streptaviden tetramer can vary, depending on conditions and desired outcome; for example, it can range from about 10^{-5} M to about 10^{-7} M, or, it can be about 10^{-6} M.

Biotinylated nucleic acid probes can also be added either to the polymerization mixture, or after polymerization of the acrylamide-streptaviden porous matrix, by perfusion, or equivalent methods. The concentration of biotinylated nucleic acid probes can vary depending on conditions and desired result; for example, the concentration can range from about 10^{-4} M to about 10^{-7} M, or, about $1x10^{-6}$ M to about $5x10^{-6}$ M, or equivalents.

In another embodiment, the substrate surface comprising an external substrate surface and a plurality of internal pores is made by contacting the porous silica matrix with an activated silane, or equivalent, by a gas phase or fluid phase deposition. A source of porous silica matrix can be, e.g., controlled pore glass (CPG) (CPG, Inc., Lincoln Park, NJ). The activated silane can be in an about 10 to 50% v/v in organic solvent, such as methanol or xylene or equivalent. In one embodiment, the gas (vapor) phase deposition method comprises mixing activated silane to about 1:1 to 1:4 in xylene contained in small, partially open vessel (such as glass petri dish) in a vacuum oven. Substrate material to be derivatized is placed in the oven and silane is vapor deposited under vacuum and elevated temperature (typically 80^0 C) for about 8 to about 48 hours. In one embodiment, the fluid phase deposition method comprises preparing activated silane as a 10% solution (v/v) in methanol. Substrate material to be derivatized is immersed in this solution for about 10 to about 30 min. After silanization, substrate is rinsed thoroughly with pure methanol and subsequently cured at about 100^0 C in an oven.

Alternatively, the Modified nylon membranes may be obtained containing amine and carboxylate groups for covalent coupling of proteins, peptides, and

oligonucleo A "tunable" porous surface can then be ma[redacted]ing the abovementioned strategies

Controlled pore glass, as mentioned above, is available with a variety of surface coatings including aminosilane, biotin, streptavidin, and many more. These functional groups may then be employed to incorporate the abovementioned "tunable" strategies

Another embodiment includes a method for detecting single base difference in a target area of a strand of DNA or RNA, comprising, mixing any of the association/ hybridization devices of the present invention with DNA or RNA containing the target area to be detected, allowing sufficient time for the probe on the association/ hybridization device to associate/ hybridize to the target area of the DNA or RNA, altering the environment of the associated/ hybridized probe/DNA or RNA, and detecting the probe/DNA or RNA remaining after the alteration. Although a variety of methods are available for altering the environment, one common method is to wash the bound probe/DNA or RNA with a solution having the specific characteristics to induce the change. For example, this could include pH changes (e.g., a pH above the pI of the composition (or net pI if more than one composition) comprising the association surface to create a net negatively charged surface on the device), ionic changes (e.g., below physiologic conditions, e.g., 2 to 20 mM sodium phosphate) and other conditions (e.g., temperature changes, or, hybridization stabilizing or destabilizing compositions in the wash solution).

Another embodiment is a method and device to obtain a precise balance of surface charge and/or charge switching ("tuning") during association/ hybridization and washing; and/or surface water binding by using parallel combinatorial screenings of compounds for hybridization surfaces. In a specific embodiment, this includes the use of amino acid derivatives as association/ hybridization surfaces.

In nucleic acid association/ hybridization analyses involving oligonucleotide probes covalently tethered to a two dimensional surface, the rate (kinetics) and specificity of specific associations (e.g., Watson-Crick type duplex or Hoogstein triplex formation) is affected by the surface itself (i.e., the localized environment). This effect may be exploited

to accent[redacted]he thermodynamic consequences of subtle, [redacted]e base target-probe mismatches in a variety of underlying substrate materials. The present invention provides the ability to modulate this surface effect to enhance duplex formation.

Because molecular biology techniques are increasingly becoming formatted into highly parallel, array-based systems using sophisticated microelectronic detection methodology to increase the speed and throughput of data acquisition, it has become increasingly important to be able to control the reaction. For the information output of any array-based association/ hybridization strategy to become meaningful, it can be helpful to understand in explicit detail all parameters affecting the thermodynamic stability of probe-target duplexes. This includes the altered reactivity of complementary nucleic acids at or near a solid-liquid interphase transition. In some circumstances, only by taking into account the above mentioned boundary conditions will perfectly matched duplexes be feasibly distinguished from relatively stable yet mismatched target-probe pairings.

The present invention is a rational use of solid support materials and the chemical alterations of such materials such that the surface becomes an active participant in the association/ hybridization process in a predictable and beneficial manner. This invention encompasses the following:

1. The ion dependence of duplex formation at the surface (i.e., localized environment) differs significantly from that in bulk solution and is selectively destabilizing for mismatched duplexes at low cation concentrations.

2. Independent of general surface physical phenomena, the chemical nature of the surface may be modified quite readily to provide surfaces differing in the nature of their electrostatic charge, hydrophobicity, density of probe molecules per unit area, tether length, and other characteristics. Further, the surfaces may be chemically modified such that the physical manifestations are "tunable" by altering bulk solution parameters (e.g., pH, temperature, and bulk cation concentration, or inclusion of other compositions).

3. The ability to chemically modify device surfaces with desirable physical properties with regard to association/ hybridization rate enhancement and/or specificity can be performed in a combinatorial fashion. One skilled in the art will

understand from the description herein that this screening methodology allows for the detection of various association/ hybridization surfaces in the shortest amount of time. In one specific example, the use of a variety of amino acids is tested by using the methyl esters of the amino acids. Since these methyl esters are commercially available, the favorable effects of these exemplary compounds is readily explored with the combinatorial methodology.

One of the advantages of the present invention is that there is a device and a method which can be used for association/ hybridization of probes to target DNA or RNA at low bulk ion concentrations. This method is effective because the surface loading of cations on a solid support creates a hybridization surface that results in a high local cation density near the surface. This localized high cation density can be used to obviate target folding and side reactions. Thus, the present invention contemplates a device in which the support surface and hybridization surfaces interact to form a high local cation density for facilitating the binding of a probe to a target nucleic acid (e.g., DNA, RNA) sequence.

One specific advantage of the present invention is that the electrostatic field created on the surface of a solid substrate by the association/ hybridization surface can be used to enhance the selectivity of duplex binding due to the interaction between the mismatches in the target, the probe and the electrostatic field of the surface.

Another benefit of the present invention is that a surface field is created which is switchable, or "tunable." This ability to switch the surface charge allows for a surface with a local ion electrostatic field which attracts the nucleic acid (e.g. , DNA or RNA) during the association/ hybridization stage yet can be altered such that local ion electrostatic field changes during the washing phase to a net negative charge density (to "repel" negatively charged nucleic acid). Thus, the probe cannot selectively interact with nucleic acid with base pair mismatches (cannot provide preferential binding). The charge "switching" also promotes disassociation among the mismatched target sequences. In this procedure, the target sequence which has the fewest mismatches with the probe will preferentially bind to the probe.

Another advantage of the present invention is that salmine A1 and its derivatives either used alone or as part of a covalent probe complex will obviate the requirement for exogenous cations in an association/ hybridization assay.

Another crucial aspect of the present invention is that by the use of combinatorial methods the device or the methods of the present invention can be fine-tuned. For example, the surface of the device of the invention can become a cationic attractor during association/ hybridization and a negatively charged discriminator during washing. In one aspect of the present invention, the method employs a combinatorial surface chemistry using amino acid derivatives as the surface chemistry. It should be noted that a combinatorial surface chemistry which generates a plurality of compounds for the association/ hybridization surface is especially "attractive" (i.e., having a net cationic charge density) when using amino acid derivatives (e.g., the pI of primary amines is 10).

In a specific embodiment, aminated polystyrene is coated as a thin layer of succinic acid on a solid surface substrate of a device of the invention. By reaction with succinic anhydride a probe will be linked to this surface by amide bond formation between an amine modified probe and the carboxylated surface. Remaining (unused) carboxylates can be modified with the methyl ester of amino acids employing the same carboxylic acid coupling used to attach the probe. In alternative embodiments, modified amino acids are added in pairs so to create surfaces with all possible mole ratios of surface chemistries (i.e., carbohydrate, amino acid 1, amino acid 2, etc.). Because of their commercial availability, the O-methyl esters of the amino acids were chosen as an example to demonstrate this principle. The standard amino acids can be varied to obtain the desired surface physical chemistries. Various amino acids and their characteristics (e.g., pIs) are well known to those skilled in the art. By the use of these methods of a variety of association/ hybridization devices can be developed, made and used.

An additional embodiment includes a method of making a library of association/ hybridization devices. One method to generate the library of association/ hybridization devices, involves using association/ hybridization devices each having a substrate surface of glass and an oligonucleotide probe linked to the substrate surface by an

epoxysilane linkage to a terminal amino modification to form a substrate surface-probe complex wherein said complex forms an effective association/ hybridization surface. The method comprises the step of converting unreacted epoxysilane groups to the corresponding cationic, neutral or anionic derivative by treating said epoxysilane groups with a reaction compound selected from the group consisting of, an amino acid, an amino acid ester and mixture thereof.

Another method to generate a library of association/ hybridization devices involves using association/ hybridization devices each having a substrate surface of glass and an oligonucleotide probe linked to the substrate surface by an epoxysilane linkage to a terminal amino modification to form a substrate surface-probe complex wherein said complex forms an effective association/ hybridization surface. In this method the unreacted epoxysilane groups are converted to the corresponding cationic, neutral or anionic derivative by treating said epoxysilane groups with a reaction compound consisting of an amino containing chemical compound.

A further method to generate a library of association/ hybridization devices, includes using association/ hybridization devices each having a substrate surface selected from the group consisting of polyvinyl, polystyrene, polypropylene and polyester, a hybridization surface of carboxylic acid placed on the support surface, an oligonucleotide probe modified to include a compound selected from the group consisting of an amino acid, an amino acid ester and mixture thereof. The modified oligonucleotide probe is reacted with the carboxylic acid group to covalently link the probe to the association/ hybridization surface.

A library of association/ hybridization devices with multiple association/ hybridization surfaces is generated in a glass bottom microtiter plate by treating each well separately with a reaction compound. Thus, each well can provide a different association/ hybridization surface if it is treated with a different reaction compound or mixture of reaction compounds.

An additional embodiment includes a method to screen the activity of a library of association/ hybridization devices, comprising the steps of mixing the library with nucleic

acid (e.g. DNA or RNA) containing a target area to be d d; allowing sufficient time for the target area to associate/ hybridize to the library of association/ hybridization devices; removing the non-associated or non-hybridized nucleic acids (e.g., DNA or RNA); and detecting the nucleic acid (DNA or RNA) hybridized to the association/ hybridization surface.

Another embodiment includes a library of association/ hybridization devices comprising a plurality of oligonucleotide probes; and a plurality of solid substrate, wherein each solid substrate has a support surface with a neutral or negative electrostatic field and a association/ hybridization surface wherein each association/ hybridization surface is accessible for linking one of the plurality of oligonucleotide probes to said solid substrate and wherein said oligonucleotide probe is linked to the association/ hybridization surface of the solid substrate at a distance of no more than about 100 angstroms. One preferred embodiment of the library includes a microtiter plate where each well in the plate is a different association/ hybridization device (e.g., has a different "association surface" but the same solid support surface).

Array Devices

The invention's charge-adjustable, or "tunable" device surfaces and methods of the invention can be adapted to any array-based hybridization detection devices, also called "genosensors," or (miniaturized) "DNA chips," or "biochips" and the like. Such devices and their applications are well known in the art, including, e.g., as described in U.S. Patent Nos. 6,093,370; 6,087,112; 6,087,103; 6,087,102; 6,083,697; 6,080,585; 6,054,270; 6,045,996; 6,022,963; 6,013,440; 5,959,098; 5,856,174; 5,770,456; 5,700,637; 5,556,752; 5,143,854; or any combination or variation thereof. See also Kurian (1999) J. Pathol. 187:267-271; Gerhold (1999) Trends Biochem. Sci. 24:168-173; DeRisi (1999) Curr. Opin. Oncol. 11:76-79; Wallace (1997) Mol. Med. Today 3:384-389; Yershov (1996) Proc. Natl. Acad. Sci. USA 93:4913-4918.

The device can also comprise other ancillary functions, such as, e.g., tubes or channels which allow for the transportation and flow of the test sample in solution, reagents, wash and hybridization buffers, and the like (see, e.g., U.S. Patent Nos. 6,010,608;

5,843,767); [redacted]mple manipulation, e.g., mixing, pumping, direction (i.e., valves) and the like; detection of compositions (e.g., fluorimetry, mass spectrometry (see, e.g., U.S. Patent No. 5,547,835), photospectrometry, detecting charged or radioactive labels, and the like, see, e.g., U.S. Patent Nos. 6,020,208; 5,209,919; computer software and hardware for programming, e.g., robotic functions for application test sample, washing, detecting signals, harvesting nucleic acids from test samples specifically associated with probes, and the like. Fluorescent probes can be detected with a variety of imaging systems known in the art (see, e.g., U.S. Patent No. 5,631,734). For example, in many commercially available microplate readers a light source (e.g., lamp or laser) is placed above a well and a photodiode detector is placed below. A camera and imaging optics can also be used. A computer can also be used to transform this data (e.g., fluorescent intensity) into various formats, calculating kinetics and binding affinities, and the like.

Kits

The invention provides kits comprising the "tunable surface" devices of the invention. In alternative embodiments, the kits further comprise reagents for practicing the methods of the invention. These reagents include buffers; including, e.g., hybridization buffers comprising pH sufficiently acidic to induce a cationic (positive) surface charge density in the device surface; and, a wash buffer sufficiently basic to induce an anionic (negative) surface charge density. The hybridization buffer can also be a low salt or no salt buffer; and, the wash buffer can comprise physiologic levels of sodium ions, or equivalents.

The kits can further comprise printed matter, e.g., written instructions for using the devices and practicing the methods of the invention.

EXAMPLES

The following Examples are offered by way of example, and are not intended to limit the scope of the invention in any manner.

Example 1: Probe Coupling Substrate Chemistries

Figure 2 illustrates covalent and non-covalent probe immobilization methodologies which were employed for surface hybridization modeling studies.

Figure 2A demonstrates the use of strepta coated microtiter plate wells. In this procedure a streptavidin monolayer hybridization surface was produced by an aqueous deposition of a dilute solution of the protein to the polystyrene wells. Probes were modified by attaching biotin to the probes. The modified probes were then attached to the hybridization surface by the non-covalent biotin-streptavidin interaction.

Figure 2B demonstrates the use of aminated plastic surfaces. In this procedure the polystyrene was aminated by gas-phase plasma amination. Surface amines were converted to the carboxylic acid by reaction with succinic anhydride. Amine-modified probes were linked to the surface by amide formation, mediated by EDC and HSSI.

Figure 2C demonstrates the use of epoxysilanized SiO_2 surfaces. In this procedure an epoxysilane monolayer was affixed to $Si0_2$ by vapor deposition. The probe was linked to the monolayer by secondary amine formation to amine-modified probe.

Example 2: Ionic Strength Dependence of Duplex Formation Near a Surface

The ionic strength dependence of DNA:DNA or RNA:DNA or other nucleic acid hybrids near any of the modified surfaces described in Example 1 is greatly reduced as compared to solution state thermodynamics. Both solution state counterion condensation theory and solution state experiments were performed. The results, as expected from known data, demonstrate that, in solution, the log of the association constant (K_a) varies linearly with the log of ionic strength over a large range. In contrast, for duplexes formed near the surface, the ionic strength dependence is flat over a large range and then association constants descend in a mismatch specific manner at lower mM Na^+ concentrations. This can be seen in Figure 3.

Figure 3, represents several possible matched and mismatched probe-target pairings from the K-ras oncogene codons 12 and 13. From Figure 3 it is readily apparent that:

1. The cation dependence of duplex formation is very shallow over the higher Na^+ concentration range (approximately 1 M to 100 mM) and then begins to lower the K_a values for binding in a manner consistent with the severity of the mismatch in the range between 100 and 10 mM Na^+.

The rank order of mismatched base pairings is consistent with known thermodynamic stabilities (i.e., Perfect match > G_A type mismatch > other non-G type single base mismatches > double base mismatches). At the lowest ionic strength tested (approximately 10 mM), the K_a for the perfectly matched pair has not diminished noticeably, and is 10 to 50 fold higher than any other surface bound duplex. This result of excellent specificity which is found in the present invention is quite surprising since the common accepted theory predicts that duplexes of this length should become unstable and disassociate at these low Na^+ concentrations.

The effect seen in Figure 3 has been demonstrated with many other surface duplex pairings. For example, epoxysilane-coated glass or SiO_2, carboxamide derivatives of aminated polystyrene, carboxamide derivatives of aminated polypropylene, other carboxylic acid derivatives of plastic, haloacetic acid derivatives of aminated plastics, plastic coated with neutral or negatively charged polymers such as the modified agaroses, organic thiol coated platinum, gold or other metals, organic acid coated gold, platinum or other metals or streptavidin or neutravidin coated plastic, glass or metal. Although the exact mechanism is not known, the following characteristics of the system are consistent with theoretical and experimental knowledge:

1. The surface itself, being inherently negatively charged or becoming so after the coupling of negatively charged probe molecules, coordinates Na^+ ions such that the effective molar concentration of cations near the surface is much greater than that in bulk solution. This phenomenon was previously unknown for nucleic acid surface hybridization.

2. Upon lowering the bulk solution cation concentration, the negative electric field manifest by the surface becomes revealed, as a loss of screening cations results in an electrostatic force extending a distance consistent with known Debye-Huckel parameters. At the lower ionic strengths where large effects are seen for the duplex pairings, the predicted field would extend to include the region containing target-probe pairs. Thus the field itself would be adding an additional electrostatic force repulsing mismatched duplexes, thereby leading to greater specificity as seen in Figure 1. This phenomenon was previously unknown for nucleic acid surface hybridization.

3. As the observed equilibrium values are dependent on both forward and reverse rate constants, it may be that the mean resident time of bound targets at the surface is greater than that in solution. As a matter of physical reality, this must almost certainly be true in that the number of possible dimensions in which a target may diffuse without encountering another probe is limited to essentially one (up). In addition, the probe, being immobilized at the surface, is not free to diffuse to any appreciable extent. Thus, the equilibrium effects described above in 1. and 2. are principally manifested through the resonance time (i.e., off rate) of the surface bound target.

Example 3: Field Effects Manifest Near a "Tunable" Surface (Streptavidin)

To demonstrate the effects of underlying charge with respect to duplex formation near a surface boundary, streptavidin modified polystyrene was used to link an oligonucleotide probe to the hybridization surface of the solid substrate. The binding of biotinylated oligonucleotide probe is very tight (K_d approx. 10^{-15} M), and due to the paracrystalline nature of these surfaces, the probes may be precisely positioned at 50 Angstrom intervals (the physical separation of biotin binding sites on the streptavidin). More importantly, the streptavidin protein has a pK of about 5.5, thereby yielding an ionizable surface which can be modulated by subtle changes in the pH of the hybridization buffer solution. Thus, at neutral pH the surface will be negatively charged; as the pH is lowered the charge on the streptavidin becomes more electroneutral to positive. It should also be noted that there is no effect on duplex formation in the range of pH 5 to 9 in solution.

The equilibrium values as a function of pH and the dissociation kinetics were examined. The association rates for a number of probe-target duplexes were measured and found to be very similar, as would be predicted for a diffusion-limited process.

Figures 4A, 4B, 5A and 5B illustrate the dramatic effect on the dissociation kinetics of matched and mismatched duplexes caused by changing the pH from neutrality to close to the pK value of streptavidin. In fact, there is no appreciable dissociation at all at the lower pH at any ionic strength. In contrast, at neutral pH, dissociation occurs in a mismatch specific manner and is sensitive to ionic strength conditions. Remarkably, it was found that specific duplex formation can occur at the lower pH on streptavidin in distilled water where

the surface i tral to cationic. One skilled in the art readil ognizes that this result is contrary to expectations, since normally under the above conditions of low salt in solution, duplex formation cannot occur.

Example 4: Hybridization on Surfaces at Low Ionic Strength as a Means f Negating the Effect of Target Secondary Structure

In addition to achieving greater than expected binding and selectivity at extremely low ionic buffer conditions, the effect of secondary structure of large target molecules (intramolecular base pairing), which in itself is ionic strength dependent, is overcome by hybridization on modified surfaces at low bulk cation concentrations.

To address this possibility, a synthetic target which is self-complementary on each end (forms a hairpin) was designed such that if the target folds upon itself, the probe binding site is obscured. This is shown schematically in Figure 6.

The results from this assay are shown in Figures 7A and 7B. As can be seen in Figure 7A, there is a two to three fold increase in binding of the target at lower ionic strengths (0 to 5 mM Na^+) than at the higher ionic strengths. In addition, the binding shows ordinary dissociation kinetics indicating that the signal obtained is not due to non-specific adsorption of targets. Figure 7B also indicates that there is excellent specificity even at equilibrium with regard to a mismatched probe. These data establish that surface effects can allow for target binding under conditions which minimize folding artifacts in solution.

Example 5: Histidine Modification of the Surface

Another class of modification is represented by the amino acid histidine. Histidine is interesting in that its pK value is around 6.7. At pH values of around 8, its side chain charge is neutral. However, at pH 6.0 its side charge is a cation and assumes a formal positive charge. Therefore, if a negatively charged surface is modified with histidine or its methyl ester at a pH lower than 6.5, the surface will become positively charged, and become a general attractor for nucleic acid targets. If during washing the pH is raised to pH 8.0, the surface will become more negative and will electrostatically repel non-specifically bound targets. In this fashion, the surface acts as a hybridization rate enhancer and a discrimination enhancer during washing. This is, as mentioned, only one example of a potential surface

which ha[redacted]n further interesting properties in addition se already mentioned above. It is a good example of a switchable electrostatic surface useful in the present invention.

Example 6: Protamine Binding

Salmine A1 was used to assess the effect of protamine binding on the stability of matched versus mismatched base pairing.

The ability of Salmine A1 to enhance binding by measuring the association constant for formation of 13 bp test duplexes, at 25°C in the presence of increasing concentration of the 31 amino acid (aa) peptide Salmine A1, or Na^+ for comparison. Binding affinity for matched and mismatched duplexes was measured.

The base pairing selectivity afforded by Salmine A1 in aqueous solution was measured. This was done by a competition method. Briefly, a biotin-modified nucleic acid probe is linked to the surface of a microtiter well, by biotin-streptavidin coupling. Up to 96 different probes can be linked concurrently in this way, employing a BioMek™ robot. The 96 well competition assay only consumes 1 nanomole of Salmine A1. Thus, not very much material is needed for multiple assays. 50 uL of complementary target was added in solution at $5x10^{-10}$ M, which upon binding to the surface, was generated accurate surface binding data when detected by chemiluminescence (AP/Lumiphos 530™).

Solution state binding equilibria were obtained by adding probe to the target solution (without biotin), or a probe homologue with base sequence changes. Duplex formation in solution consumes free target, thereby reducing the amount of target bound to the surface at equilibrium. By monitoring surface binding while performing a solution state probe titration, an accurate binding isotherm was obtained, to yield the association constant for duplex formation.

Large libraries of 9, 11, 13 and 19 base pair (bp) duplex pairs were synthesized and analyzed in the above microtiter format. For the Salmine A1 analysis, a 13 bp assay set was employed. This set focused on the set of 32 duplex pairs described below. This set allowed the observation of the effect of Salmine A1 on all mismatches, flanked by AT or GC base pairs.

13 BP TEXT DUPLEX

5'-CTGGCGGAAXATC-3' AT RICH FLANK

3'-GACCGCCTTYTAG-5'

5'-CTGGXGGAATATC-3' GC RICH FLANK

3'-GACCYCCTTATAG-5'

wherein X is A,T,G or C and Y is A,T,G or C.

In Figure 9A, the solution state association constants are displayed as a function of Na^+ ion concentration for 13 bp duplex formation. The perfectly matched (PM) 13 mer duplex displays approximately a 30-fold increase in association constant per 10-fold Na^+ ion concentrate in the range from 1.8 to 180 mM, as expected from published theory.

Duplexes bearing a single CA or GA mismatch display affinities which are reduced by a factor of 50 relative to the perfect match, at all Na^+ concentrations greater than 18 mM (solid bars).

By comparison, in Fig. 9B, solution state duplex formation in the presence of Salmine A1 saturates at approximately 1000 fold lower ion concentrate than for Na^+. Over the range from 30 nanomolar to 1 mM of added Salmine A1. The solution state association constant for mismatched duplex formation is at least 1000-fold lower than for the perfectly matched 13 bp duplex. The selectivity is sufficiently high that mismatched duplex formation cannot be detected experimentally at Salmine A1 concentrations lower than 10 uM.

The results suggest that Salmine A1 can drive high affinity double helix formation in solution at sub-micromolar concentration, and that in this range, the selectivity of duplex formation has been increased 20-50 fold relative to that seen when Na^+ ion is used to drive duplex formation.

Example 7: Effect of Amino Acid Sequence Change on Protamine Binding

The known members of the protamine family, are described by the following consensus sequence:

SALMINE A1:

Pro-Arg-Arg-Arg-Arg-Ser-Ser-Ser-Arg-Pro-Val-Arg-Arg-Arg-Ag-Arg-Pro-Arg-Val-Ser-Arg-Arg-Arg-Arg-Arg-Gly-Gly-Art-Arg-Arg-Arg-COOH

CONSENSUS: $[arg)_n\text{-bend}]_m\text{-arg}_n$

wherein n=3-5, m=3-5 and bend = 1-3 amino acid sequence capable of inducing a "kink" in the polypeptide backbone (pro, gly-gly, etc.)

The 32 aa sequence of Salmine A1 easily falls into this consensus. One skilled in the art readily recognizes that the use of symmetrical derivatives of SalA1 is a design improvement. The symmetric derivatives can be synthesized blockwise much more inexpensively than a unique 25-35 aa arginine-rich peptide of this kind.

Synthetic peptides are synthesized in small quantities, first varying the nature of the kink or bend in the molecule, then the span of the oligo-arginine domain.

First generation peptides:

$[(arg)_5\text{-pro}]_5\text{-arg}_5$	23 aa rigid bend
$[(arg)_5\text{-pro}]_4\text{-arg}_5$	29 aa rigid bend
$[(arg)_5\text{-pro-gly}]_3\text{-arg}_5$	29 aa semi-rigid bend
$[(arg)_5\text{-pro-gly}]_4\text{-arg}_5$	33 aa semi-rigid bend
$[(arg)_5\text{-gly-gly}]_3\text{-arg}_5$	26 aa flexible bend
$[(arg)_5\text{-gly-gly}]4\text{-arg}_5$	33 aa flexible bend

Subsequent to identifying optimal bend above, the length of the oligo-arginine motif is varied: $[(arg)_3\text{-bend}]_3\text{-arg}_3$ $[(arg)_4\text{-bend}]_3\text{-arg}_4$ $[(arg)_5\text{-bend}]_3\text{-arg}_5$

Example 8: Rational Design of Active Surface Films

When duplex formation is constrained to occur within 100 angstrom (A°) of the solid support, solid phase nucleic acid hybridization will be affected by surface effects. However, until the present invention, there was no published data which addressed the role of surface effects in nucleic acid hybridization, or how such surface effects might be exploited.

It is known that polystyrene can be coated with streptavidin. Streptavidin forms a paracrystalline monolayer, with sites for biotin binding positioned 50 angstroms apart. Oligonucleotides are readily synthesized with a biotin group at the 3' terminus.

Streptavidin d polystyrene was used as a prototype for t[redacted]dy of orderly surface films, to which probes can be affixed with spatial precision for the purpose of quantitative nucleic acid hybridization analysis. Solution state nucleic acid targets were synthesized with digoxigenin (D) at the 3' terminus, so that duplex formation with probe oligomers on the streptavidin film support were monitored by anti-digoxigenin-AP chemiluminescence. A 96 well microliter format was employed, assayed with an EGG robotic luminometer.

19 BP

PM 5'--TGACTGGCGGAATATCTGT-3'B probe
D3'-ACTGACCGCCTTATAGACA-5' target

CA 5'--TGACTGGCGGAACATCTGT-3'B probe
D3'-ACTGACCGCCTTATAGACA-5' target

GA 5'--TGACTGGCGGAAGATCTGT-3'B probe
D3'-ACTGACCGCCTTATAGACA-5' target

GT 5'--TGACTGGCGGAAGATCTGT-3'B probe
D3'-ACTGACCGCCTTTTAGACA-5' target

B = BIOTIN and D = DIGOXIGENIN

Figure 10 displays the chemiluminescent signal due to bound target (in relative luminescence units) versus the Na^+ ion concentration used during hybridization and washing. Samples were hybridized for 12 hours at 25°C and pH 7.2, which was sufficient to reach binding equilibrium. Target concentration was held constant at $5x10^{-10}$M (strands). Probe density is fixed at $4x10^{10}$ molecules per mm^2, which constitutes saturation of the available biotin binding sites (roughly 1 probe per 50 angstroms on center).

With a target with a perfectly matched 19 mer duplex (top curve), little or no Na^+ ion dependence was seen over the range from 20 mM to 200 mM and above. This effect is not due to saturation of available probe sites on the surface (less than 1% of probes are bound to target in this assay) and has been seen for all duplexes in the 10 to 20 bp range.

Further, none of the signal obtained in these experiments can be ascribed to trivial effects such as surface absorption, since targets with double mismatches do not yield binding signals above background.

Therefore, the data suggest that the ionic s[redacted]th dependence of duplex formation on the orderly streptavidin coated surface is quite different than that of double helix formation in solution.

Upon reducing bulk Na^+ concentrate to 10 mM, an abrupt 3 fold diminishment of target binding is detected (Fig. 10), indicative of a physical discontinuity in the binding process in the 10-20 mM range. Since streptavidin is negatively charged at pH 7.2, the paracrystalline streptavidin surface film must produce a negative surface potential. Standard Gouye-Debye theory predicts that this surface potential should decay exponentially, with a 1/e length near to 30 angstroms at 10 mM. Since that length is near to the separation between the surface film and the bound duplex, the Na^+ ion discontinuity detected below 20 mM is a direct result of the interaction between the surface field and the bound double helix.

The lower curves of Figure 10 correspond to binding data for targets which yield a single CA, GA or GT mismatch in the 19 mer duplex. The single mismatches cannot be detected in such 19 mer duplex above 100 mM of Na^+ ion, the standard Na^+ range for hybridization analysis of duplexes of this size at 25°C.

However, upon reducing the Na^+ ion concentration below the point of discontinuity at 20 Mm, single base mismatches can now be detected in a 19 mer duplex, giving rise to a clear 5-fold signal differential. This mismatch discrimination is maximized below 20 mM Na^+, for duplexes in the 9 to 19 mer range.

Example 9: Streptavidin Binding

At low ion concentration, interaction with the surface electrostatic field is an explicit source of target binding selectivity. This was further demonstrated using streptavidin which has a pK of about 5.5 Since double helix stability has little pH dependence in solution between pH 5 to 8, target binding was measured at pH 5.2, where the streptavidin film is nearly neutral and at pH 7.2, where the surface potential due to streptavidin is large and negative. The kinetics of target dissociation were monitored by employing the 12 bp test duplex and the highly quantitative chemiluminescence assay.

In this kinetics experiment, target binding was allowed to reach equilibrium during the "hybridization" phase of the assay at 50 mM Na^+, pH 7.2. Dissociation kinetics

were monitored with vigorous rotary mixing over 180 min during the "washing" phase by changing the buffer to 5 mM Na^+ at time zero, at either pH 7.2 (Fig. 4A), or pH 5.0 (Fig. 4B).

Target controls which yield a doubly mismatched duplex do not yield measurable signals in this kinetic assay, therefore all signals in this kinetic assay are due to oligonucleotide-oligonucleotide binding.

In these kinetics, the zero time intercept corresponds to the outcome of the binding equilibrium obtained at 50 nM Na^+ pH 7.2. As would be inferred from the 19 mer data (Figs. 10A and 10B), the CA, but not the GA mismatch was discernable from the perfect match at this "high salt" equilibrium condition. At neutral pH (7.2) (Fig. 4A), substantial dissociation kinetics, with rates which are roughly proportional to the equilibrium value detected at time zero ($R_{CA}>R_{GA}>>R_{PM}$) were observed.

From a practical point of view, the data show that the selectivity of duplex formation increases steadily with time, the ratio of matched versus mismatched target after a 180 min "wash" being 10 fold greater than at equilibrium.

More fundamentally, the data suggest that at 5 mM Na^+ ion concentration and pH 7.2, the mean residence time of the duplex upon the surface has become dependant upon single base pair mismatches.

In principle, this kinetic distinction could be due to interaction within the double helix, or to the interaction of the helix with the surface field. In order to separate these two possibilities, we have repeated the kinetics experiment, keeping Na^+ ion fixed at 5 mM, but lowering the pH of the wash solution to pH 5.2. Here, the streptavidin surface should become nearly uncharged, but the internal stability of the duplex should remain unaffected.

Figure 4B shows that surface neutralization has an enormous effect on duplex dissociation rate. The rates have become too slow to measure for both matched and mismatched duplexes upon the neutralized streptavidin surface. The kinetic data therefore indicate that it is the electrostatic interaction with the surface, and not internal duplex electrostatics which dominate the kinetics of short duplex formation at pH 7 upon streptavidin coated polystyrene.

Thus, the electrostatic field near the solid [redacted]ort plays an active role in defining the affinity and selectivity of double helix formation in hybridization. The skilled artisan will readily recognize that the streptavidin model is not unique in this regard.

The skilled artisan will readily recognize that a variety of compositions are useful as hybridization surfaces. Examples of some useful compositions are shown in Table 1 and Figures 12 to 17.

Example 10: Rational Design of Active Surface Films

In addition to streptavidin, chemically simpler surfaces were prepared which display similarly useful physical features. A 20 ml polypropylene was plasma aminated to 0.5×10^{-9} moles/cm^2. This is a durable, high quality substrate, which is available commercially in large quantities.

Several surface chemistries listed in Table 1 were tested. All are consistent with high efficiency attachment of amine-modified oligonucleotide probes.

Table 1. Activation of Amines

	Name	Ion & pH	Fig. No.	Description
1.	Streptavidin coated surface	Anionic pH 7.0	12	The bond formation between biotin and streptavidin is very rapid and once formed, is unaffected by m st extremes of Ph, organic solvents and detergents.
2.	Succinic anhydride	Anionic pH 7.0	13	Succinic anhydride is a good acylating agent, and commonly used to immobilize biopolymers onto a surface through an amide bond.

	Name	Ion & pH	Fig. No.	Description
3.	Cyanogen bromide	Neutral pH 7.0	14	Cyanogen bromide is a versatile reagent has been widely used in activation of solid matrices containing hydroxyl and amino groups. It reacts with these surfaces quickly to form a reactive group which is susceptible to nucleophilic attack by an amino group and results in a guanidine linkage.
4.	. α-Haloacids	Neutral, Zwitterion pH 7.0	15	. -Haloacids are very reactive and are commonly used alkylating agents. The reactivity of the haloacids is a function of the halogen in the order of I>Br>Cl. Alkylation of amines with haloacids is one way of converting amines to their corresponding carboxylic acids. . -Haloacids such as iodoacetic acid or bromoacetic acid alkylates nucleophiles, such as an amino group, to give corresponding acid derivatives ,-Unsaturated acids such as acrylic acid and bromoacrylic acid are known to react with primary amines to give corresponding higher homologues.
5.	Ethylene oxide	Cationic pH 7.0	16	Conversion of an amine group t an hydroxy can be achieved with ethylene oxide. Oxiranes will be converted to alcohols then can be converted to good leaving group.

	Name	Ion & pH	Fig. No.	Description
6.	Epichlorohydrin	Cationic pH 7.0	17	Epichlorohydrin activates matrices with nucleophiles such as amino r hydroxyl to an epoxide derivative. This epoxide derivative reacts with nucleophilic amino group.

These surface chemistries are employed as a linker to the oligonucleotide and as a vehicle to alter the surface properties of the plastic substrate. At 5×10^{-9} moles/cm^2, the primary amines on the plastic surface are positioned approximately 7A° apart. Upon coupling to the appropriate linker, a nearly uniform surface film results. Probe coupling is regulated so that only 1% of these linkages are consumed, the remaining 99% being unaltered in their chemical properties.

With this procedure, three classes of surface film can be obtained: cationic, neutral zwitterionic and anionic at pH 7. The nominal pK of the groups described in the accompanying table is far from neutrality. However, in preliminary results the apparent pK of such densely-packed surface films may deviate greatly from that of the linker in isolation, presumably due to the fact that, at 7A separation, charged groups are constrained to be within a Debye length, especially at low bulk ionic strength.

This pK shift is studied by measuring the effect of bulk pH change on the affinity and kinetics of target binding to those surfaces in the range from pH 5 to 8. This effect is of importance in terms of basic hybridization technology.

Example 11: Amino Acid Combinatorial Approach

In Example 10, Table 1 showed the activation of amines on the solid substrate. Any of the chemistries 2-6 can be used for creating an amino acid combinatorial surface. As shown in Figures 11A and 11B, a plurality of amino acids can be attached to the surface. In the figures $R_{n,m}$ can be any two different amino acids attached to the surface. Thus, the surface can have several surface chemistries. There is a probe, a first amino acid, a

second amino acid an unmodified carboxylic acid (Case 2 in Table 1) or unmodified linker chemistry (Case 2-6 in Table 1).

Example 12: Development of an Oligonucleotide-Peptide Chimera

Salmine A1 has no primary amines and a single carboxylate group at its C-terminus. Therefore, it can be linked, uniquely, by standard aqueous carboximide chemistry to a terminal group synthesized onto an oligonucleotide: Pro-SAL-A1-CONH-OLIGO.

Chimera of this sort are purified by Sephadex G100™ chromatography in 6 M GuHC1. The identity of the final complex is confirmed by ESMS. The ability of such chimera to form SAL-A1 probe target complexes is assessed both in solution, by the competition method, and on solid supports. For solid phase binding analysis, the conjugate is linked to the surface by means of biotin coupling, using probes which bear biotin at the 3'-terminus, and a primary amine at the 5'- terminus for covalent coupling to the protamine. Such conjugates form selective double helix-protamine triple helices in the absence of other cations.

From the results shown in the above Examples, it is apparent that modified "smart" surfaces may be used to considerable advantage with regard to the selectivity of duplex formation as well as by negating the effect of target secondary structure in solution. These results have been obtained on substrate materials which are compatible with developing microelectronic detection devices (i.e., optically pure and sturdy). Some examples of these substrate surfaces include quartz, SiO_2, polystyrene, polypropylene and polyester.

The streptavidin pH "tunable" surface was representative of only one of many possible chemical modifications which can be made using existing repertoires of surface chemistries. The present invention has shown that there are developed methodologies to chemically modify a variety of surfaces in a combinatorial fashion, as well as instrumentation to insure high throughput for screening. One skilled in the art readily recognizes that there are a variety of beneficial modifications.

E le 13: Hybridization rate enhancement [redacted]ow ionic strength and low pH conditions

The following example demonstrates that the methods of the invention incorporating low ionic strength, low pH buffers (together with a net cationic charge density on the device surface) as hybridization conditions provide a significant increase in kinetics of probe:nucleic acid association (rate enhancements) for high molecular weight (MW) DNA targets ("test samples") binding to microarray probes.

DNA microarray technology has recently become a valuable tool for the large-scale analysis of nucleic acids, e.g., in gene polymorphism and the analysis of gene expression at the mRNA level (see, e.g., Collins (1999) Nature Genetics Suppl. 21:2). However, before this invention, high throughput or point of care application of microarray technologies was limited by the generally slow kinetic process by which a dilute, partially structured, high MW nucleic acid target could seek out and subsequently bind to a surface bound probe to generate a detectable duplex. To enhance the rate of target binding, electrophoretic hybridization enhancement methods have been tried (see, e.g., U.S. Patent 5,670,322; Sosnowski (1997) Proc. Natl. Acad. Sci. 94:1119). To minimize the effect of undesired secondary structure within a nucleic acid target (always a problem for high MW nucleic acid samples), modified nucleic acid probe chemistries (e.g., peptide nucleic acids, PNAs) which allow for duplex formation at low salt where secondary structure in the target would be minimized, have been tried (see, e.g., Tomac (1996) J. Am. Chem. Soc. 118:5544-5552).

As an alternative to the above approaches, the invention provides a new method for the production and use of DNA microarrays. The methods of the invention produce a major increase in hybridization rates between test sample nucleic acids (particularly high MW DNA) without the use of electric currents; while at the same time allowing for duplex formation under low salt hybridization conditions, but with standard rather than modified DNA chemistries.

These exemplary methods are, in part, based upon the invention's "tunable" hybridization surface, as described above. This example uses the embodiment wherein a positive charge is induced onto the solid support surface or association surface (of the device

of the invent during the hybridization step. Without bein ited by any particular theory of activity, the net cationic charge density of the device of the invention exploits weak, favorable adsorptive interactions with the negatively charged target DNA polyanion. Subsequently, the solid support surface or association surface is induced to assume a net negative charge density during the wash step. The nucleic acid duplexes formed during hybridization are then subject to unfavorable electrostatic (repulsive) interaction with the solid support surface or association surface of the device. This results in a significant increase in duplex selectivity in addition to an increase in the kinetics of association and disassociation. The increase in duplex sensitivity can be equivalent to that seen if there had been an increase in temperature during washing.

Microarrays were fabricated using biotin-modified oligonucleotide probes complexed with streptavidin (SA). SA binds to the biotinylated probes, thus non-covalently attaching the probes to the solid support surface of the device of the invention. As discussed below, at low salt and pH 5, where streptavidin develops a positive charge (streptavidin has a pI of 5.5), duplex formation becomes at least 80 fold faster than seen under standard conditions, where streptavidin is neutral or anionic. Duplex formation becomes independent of solution state cation concentration in the low pH state, under conditions where specificity remains high.

Without being limited by any particular theory of activity, positive device surface charge (net cationic charge density) during hybridization may be increasing the rate of duplex formation due to local concentration of target (e.g., DNA in a test sample) near the device surface (and the immobilized oligonucleotide probe). It may also cause an increase in the rate of unfolding of a partially structured or randomly coiled target (test sample) nucleic acid onto the charged probe-containing device surface.

Without being limited by any particular theory of activity, a net negative surface charge on the device during the wash step may increase the stringency of duplex formation and/ or dissolution via a general decrease of duplex melting temperature due to proximity of a negative (repulsive) surface field. Thus, the devices and methods of the invention can increase rates of hybridization and disassociation and increase stringency

without [redacted]eed to alter temperature (i.e., the device can [redacted]ptimally operated and the methods of the invention practiced at room temperature).

"Tunable surface" DNA microarrays were fabricated by first modifying oligonucleotides with a terminal biotin. Biotinylated probes were then complexed in solution with a streptavidin tetramer at a 4/1 ratio of oligomer to tetramer. This protein-DNA complex was then printed directly onto clean polystyrene and allowed to link to the surface. The adsorptive interaction between SA and a neutral plastic surface is well-known, see, e.g., Shao (1992) Anal. Biochem. 205:77.

In addition to being a useful vehicle for surface attachment, streptavidin was selected for this example because SA attached to a solid support (e.g., a neutral plastic surface) has a pI near 5.5. Therefore, SA is a highly anionic protein at neutral pH. However, SA can be driven into its cationic form below its pI of 5.5, e.g., at pH 5, while retaining its ability to bind biotin with high affinity. Equilibrium leading to nucleic acid duplex formation is not generally pH dependent in solution over the range from about pH 5 to about pH 9 (see, e.g., Sivasankar (1998) Proc. Natl. Acad. Sci. USA 95:12961-12966). Therefore, observed pH dependent effects in the range from about 5 to 9 can be linked to a change in surface charge due to ionization of the SA coated surface.

To form microarrays, polystyrene in a standard 96-well format (Corning) with raised circular wells was employed. Microarrays were printed with a Hamilton Micro Lab 2200™ robot (Hamilton Instruments). The robot deposited 25 nL of a mixture of SA (2μM) and biotin-modified oligonucleotide probes (8 μM) in 20 mM NaOAc at pH 5.5 per array spot element. Each array had 14 elements, representing 7 capture oligonucleotides printed in duplicate. Subsequent to printing, arrays were incubated overnight at RT in a humidity chamber to allow the stable adsorption of SA upon the underlying polystyrene substrate.

As a representative DNA hybridization model a 157 bp PCR fragment of the human k-ras oncogene was used. Because of its high GC content, this "amplicon" hybridizes rather poorly under "ordinary" (e.g., physiologic salt and pH, RT) conditions (possible because of undesirable intra-strand folding of the k-ras fragment in solution).

The biological significance of k-ras is related in part to point mutation in

codon 12. Therefore, in this study, oligonucleotides complementary to codon 12 mutations were designed to serve as capture probes on the array, with the following sequences: K1: 5'-gacctggtggcg-3' ; K2: 5'-gacctagtggcg-3' ; K3: 5'-gacttggtggcg-3' ; K4: 5'-gacctcgtggcg-3' ; K5: 5'-gacctgatggcg-3' ; K6: 5'-gacctgctggcg-3' ; K7: 5'-gacctgttggcg-3' .

In the current study, cell-line derived PCR k-ras amplicon targets complementary to capture probes K1, K2 and K7 were used. K-ras amplicons were amplified by the polymerase chain reaction, PCR. The PCR protocol was the following: one pre-PCR cycle 94°C for 12 min, 60°C for 1 min, and 72°C for 1 min; 35 PCR cycles 95°C for 1 min, 57°C for 1 min, 72°C for 1 min; hold cycle 72°C for 7 min, hold at 4°C. PCR primers (Midland Certified Reagent Co.) for k-ras amplicons were labeled with digoxigenin at their 5' ends during synthesis and had the following sequences: 5'-dig-actgaatataaacttgtggtagttggacct-3' and 5'-dig-tcaaagaatggtcctgcacc -3'.

The kinetics of target k-ras amplicon (the "test sample") hybridization to the probe (immobilized to the SA surface of the device of the invention) under "standard" high salt, high pH conditions was compared to hybridization under low salt, low pH conditions.

High salt, high pH prehybridization solution contained 150 mM sodium citrate (in respect to sodium ion concentration), 5X Denhardt's solution, pH 8.0. This was applied to the array for at least 10 minutes. It was vacuumed off and high salt, high pH hybridization solution (1 nM amplicon, 0.1 μM chaperone, 150 mM sodium citrate in respect to sodium, 5X Denhardt's solution, pH 8.0) was applied to the array. After hybridization, the array was washed two times in 100 mM sodium citrate with respect to sodium 10 minutes each, pH 7.8, followed by a brief rinse in 1XSSC.

Low salt, low pH prehybridization solution contained 0.2 % Tween 20 in 5X Denhardt's solution. It was applied to the array for at least 10 minutes. It was vacuumed off and low salt, low pH hybridization solution (1 nM of amplicon in 2 mM sodium phosphate, containing 0.1 % Tween 20 at pH 5.0) was applied to the array. After hybridization, the array was washed once in 100 mM sodium phosphate, pH 7.8 for 10 minutes, followed by a brief rinse in 1XSSC.

The digoxigenin-labeled amplicon was detected using anti-digoxigenin

antibody [redacted]ed to alkaline phosphatase (Boehringer Mar[redacted]m) and by an enzyme linked fluorescent substrate (ELF, Molecular Probes Inc., Eugene, OR) for alkaline phosphatase as described by R.P. Haugland, Handbook of Fluorescent Probes and Research Chemicals; Molecular Probes, Inc., ed. 6, 1996, pp.117-120. The anti-digoxigenin antibody was used at 1:1000 dilution in the blocking buffer from the ELF-97 mRNA In Situ Hybridization Kit™ (Molecular Probes Inc.), followed by wash in buffer A from the same kit and by application of ELF as described in the kit, which is a substrate for alkaline phosphatase. After being cleaved by alkaline phosphatase, ELF molecules precipitate and become fluorescent. The fluorescence intensities were detected with an Alfa Imager 2000™ apparatus and processed using GeneView 1.0™ (Genometrix, Inc., The Woodlands, TX), Microsoft Excel 97™, and Sigma Plot 3.0™ software. In both instances, hybridization and washing were performed at 25°C, to maximize the opportunity for surface interaction and for the sake of general experimental simplicity. Also, in both cases, hybridization was followed by a "high salt" wash step: i.e. 100 mM of a sodium containing buffer, pH 7.0.

Kinetic data derived from quantification of fluorescence intensities are shown in Figure 18. Figure 18A shows data demonstrating the slow kinetics of k-ras 1 amplicon hybridization at high salt and high pH conditions. Solid lines represent a probe with a perfect base pair match (k1 probe, above). Open lines represent a probe with a single base pair (a G-T) mismatch (the k3 probe); hybridization signals indistinguishable from background. All other single base pair mismatches (i.e., GA, GG, CC, CT, TC) gave hybridization signals which also were indistinguishable from background. Figure 18B shows data demonstrating the fast kinetics of k-ras amplicon hybridization at low salt and low pH conditions. Solid lines represent perfectly matched base pair of probe to k-ras target (k1 probe). Open lines represent probes with a single G-T mismatch (k3 probe); hybridization signals indistinguishable from background. All other mismatches (i.e., GA, GG, CC, CT, TC) gave hybridization signals which also were indistinguishable from background.

As summarized in Fig. 18, hybridization kinetics for a 1 nM solution of the wild type (WT) k-ras amplicon approximate a simple exponential rise to equilibrium, with a half time of approximately 40 minutes at standard high salt, high pH conditions (Fig. 18A)

and less than [redacted]econds for low salt, low pH condition (Fig. Thus, the observed hybridization rate enhancement was at least 80 fold. In the low salt, low pH condition, hybridization was found to be nearly instantaneous (relative to a 30 second time resolution of the fluid delivery system of the device). Furthermore, washing could be reduced to as little as 30 seconds. Thus, with appropriate fluidics, using the methods and devices of the invention, hybridization and washing can be completed in less than one minute.

Selectivity with respect to a single base mismatching is seen to be good for both sets of hybridization condition. Even a single G-T mismatch (Figs. 18A and 18B, open lines) generates signals which are diminished by a factor of twenty five (for fast hybridization) and by a factor of ten (for long hybridization) relative to perfectly matched 12-bp pairing. Discrimination for all other single base mismatches (i.e., GA, GG, CC, CT, TC) was measured to be significantly greater than a factor of twenty five.

Supporting data employing surface plasmon resonance suggested that for the low salt, low pH condition, the observed selectivity was, for the most part, obtained during the hybridization step, rather during washing. Duplex formation was not detected for the k-ras model or other target-probe pairs (with k-ras amplicons) when hybridization was performed at 2 mM of sodium, but at pH 7.0 on the SA surface described herein. At pH 5.0, the kinetics and equilibrium properties of duplex formation on the SA surface are not significantly affected by a ten fold increase in sodium concentration from 2 mM to 20 mM in the hybridization buffer. Together, these data demonstrate that under conditions where the SA surface of the device of the invention has been driven to assume a positive charge, the induction of positive surface charge obviated, at least in part, the ordinary hybridization requirement for screening counter-ion in solution.

It is important to note that the standard high salt, high pH k-ras hybridization was performed in the presence of a soluble "chaperone" oligonucleotide probe, which had the sequence: 5'-taggcaagagtgccttgacgatac-3'-dig. The soluble "chaperone" forms a duplex with the target (the test sample nucleic acid) immediately proximal to the target-probe hybridization site. By this binding the chaperone holds the portion of the k-ras target in a locally opened (i.e., non-folded) state to minimize undesired side effects of target secondary

structu[redacted]most no binding (i.e., background levels) v[redacted]tected in the absence of "chaperone" under those high salt, high pH conditions; thus, undesired secondary structure within the target k-ras strand inhibited hybridization to probe under high salt, high pH conditions.

However, when hybridization was done under low salt, low pH conditions, the chaperone produced no measurable change in hybridization signal. Companion experiments performed with amplicons without chaperones (i.e., amplicons which do not require a chaperone, e.g., the rat Neu amplicon) reveal an equivalent extent of hybridization rate enhancement. This demonstrates that the effect of using a device with a net cationic surface charge on DNA hybridization rate is independent of primary (sequence) or secondary (intra-strand folding) structures in the target (test sample nucleic acid).

Rate enhancement effect and high sequence selectivity by the methods and devices of the invention has also been observed in real kinetic time via the label free surface plasmon resonance technique. Rate enhancement effect and high sequence selectivity was observed using the surface and k-ras amplicon target model described above, as well as for other target-probe models which do not require a chaperone (e.g., the rat Neu amplicon). This demonstrates that the observed hybridization enhancement is a general property of the interaction between DNA and immobilized probes on the invention's device surfaces (including embodiments comprising adjustable, or "tunable," device surface charge).

One skilled in the art recognizes that the present invention provides new and novel benefits including providing teachings to modify the surface to enhance hybridization selectivity, sensitivity and discrimination power. Surface physical chemistry can be modified to play a significant role in duplex formation at surfaces, particularly with regard to ionic strength dependence. The participation of the surface in the binding reaction is exploitable by the use of active chemical modifications. Finally, using the teachings of the present invention additional modifications can be rapidly searched by using combinatorial means.

WHAT IS CLAIMED IS:

1. An association device comprising a plurality of nucleic acid probes or polypeptide probes or a combination thereof linked to a porous solid substrate;

wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end opening to the external surface to allow passage of fluid into a pore, and wherein the pore surfaces comprise an association surface;

wherein the association surface comprises a charged surface comprising net positive (cationic) charge density under conditions comprising a pH lower than the pI of the association surface;

wherein the association surface comprises a charged surface comprising a net negative charge density under conditions comprising at a pH higher than the pI of the association surface; and

wherein the distance between the nucleic acid probe or the polypeptide probe and the charged association surface is no more than about 100 angstroms.

2. The association device of claim 1, further comprising an aqueous solution comprising a pH lower than the pI of the association surface, thereby inducing a net positive (cationic) charge density on the association surface.

3. The association device of claim 2, wherein the net positive (cationic) charge density on the association surface induces a net positive (cationic) charge density in the pore space of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of negatively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of positively charged molecules out of the pore space.

4. The association device of claim 3, wherein the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides into the pore space from the aqueous solution outside of the pores.

5. The association device of claim 2, wherein the positively charged association surface comprises at least about 10^{11} charges per square millimeter, or a charge equivalent thereof.

6. The association device of claim 1, comprising an aqueous solution comprising a pH higher than the pI of the association surface thereby inducing a net negative (anionic) charge density on the association surface.

7. The association device of claim 6, wherein the net negative (anionic) charge density on the association surface induces a net negative (anionic) charge density in the pore space of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of positively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of negatively charged molecules out of the pore space.

8. The association device of claim 7, wherein the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides out of the pore space.

9. The association device of claim 6, wherein the negatively charged association surface comprises at least about 10^{10} to about 10^{11} charges per square millimeter, or a charge equivalent thereof.

10. The association device of claim 1, wherein the association surface comprises a streptavidin or streptavidin derivative linked to an oligonucleotide or a polypeptide probe.

. The association device of claim 10, wherein the streptavidin or streptavidin derivative is present on the association surface at a density of at least about 10^{10} molecules per square millimeter.

12. The association device of claim 1, wherein the association surface comprises a histidine or histidine derivative linked to an oligonucleotide or a polypeptide probe.

13. The association device of claim 12, wherein the histidine or histidine derivative is present on the association surface at a density of at least about 10^{10} molecules per square millimeter.

14. The association device of claim 1, wherein the association surface comprises an imidazole or an imidazole derivative linked to an oligonucleotide or a polypeptide.

15. The association device of claim 14, wherein the imidazole or imidazole derivative is present on the association surface at a density of at least about 10^{10} molecules per square millimeter.

16. The association device of claim 1, wherein the association surface comprises a citrate or citrate derivative linked to an oligonucleotide or a polypeptide probe.

17. The association device of claim 16, wherein the citrate or citrate derivative is present on the association surface at a density of at least about 10^{10} molecules per square millimeter.

18. The association device of claim 1, wherein the internal pores comprise a diameter of at least about 10 angstroms to about 1000 angstroms.

19. The association device of claim 18, wherein the internal pores comprise a pore diameter of at least about 50 angstroms to about 700 angstroms.

20. The association device of claim 19, wherein the internal pores comprise a pore diameter of at least about 100 to about 600 angstroms.

21. The association device of claim 20, wherein the internal pores comprise a pore diameter of at least about 500 angstroms.

22. The association device of claim 1, wherein the porous solid substrate comprises a porous bead or a porous microsphere or an equivalent structure.

23. The association device of claim 1, wherein the porous solid substrate comprises a porous membrane, a microporous membrane or a porous film or an equivalent structure.

24. The association device of claim 1, wherein the porous solid substrate forms a side or a bottom of a well in a microtiter plate or an equivalent structure.

25. The association device of claim 1, wherein the porous solid substrate comprises a porous polytetrafluoroethylene filter or a porous filter comprising an equivalent composition.

26. The association device of claim 1, wherein the porous solid substrate comprises a porous fiber, a porous hollow fiber or a porous fabric.

27. The association device of claim 1, wherein the porous solid substrate comprises a polyacrylamide or an equivalent polymer composition.

28. The association device of claim 27, wherein the porous solid substrate comprises a polymethacylamide, a methyl methacrylate, a glycidyl methacrylate, a dialkylaminoalkyl-(meth)acrylate, or a N,N-dialkylaminoalkyl(meth)acrylate, or an equivalent composition.

29. The association device of claim 1, wherein the porous solid substrate comprises an agarose or an equivalent polymer composition.

30. The association device of claim 1, wherein the porous solid substrate comprises a polyimid or an equivalent polymer composition.

31. The association device of claim 1, wherein the porous solid substrate comprises a controlled pore silica or porous glass or an equivalent composition.

32. The association device of claim 1, wherein the porous solid substrate comprises a porous foam or an equivalent composition.

33. The association device of claim 32, wherein the porous foam comprises a poly(D,L glycolic-co-lactic acid) or a poly(D, L-lactide-co-glycolide) (PLGA) or an equivalent composition.

34. The association device of claim 1, wherein the porous solid substrate comprises a porous ceramic or an equivalent composition.

35. The association device of claim 1, wherein the porous solid substrate comprises a poly(ethylene glycol terephthalate) (PEGT) or a poly(butylene terephthalate) (PBT).

36. The association device of claim 1, wherein the porous solid substrate comprises a monodispersed carbon nanotube or a nanotube comprising an equivalent composition.

37. The association device of claim 36, wherein the monodispersed carbon nanotube or a nanotube comprising an equivalent composition comprises patterned porous silicon or an equivalent composition.

38. The association device of claim 1, wherein the porous solid substrate comprises porous polystyrene or an equivalent composition.

39. The association device of claim 38, wherein the polystyrene comprises a porous poly(styrene-divinylbenzene) (PS-DVB) or an equivalent composition.

40. The association device of claim 1, wherein the porous solid substrate comprises a plastic or a plastic co-polymer or an equivalent thereof.

41. The association device of claim 1, wherein the porous solid substrate comprises a polyvinyl, a polypropylene or a polyester or an equivalent thereof.

42. The association device of claim 1, wherein the porous solid substrate comprises a poly(vinyl alcohol) (PVA) hydrogel nanoparticle.

43. The association device of claim 1, wherein the porous solid substrate comprises a plurality of different nucleic acid probes, wherein the probes are arranged in spatially defined areas over the surface of the association device.

44. The association device of claim 1, wherein the pores comprise a closed distal end.

45. The association device of claim 1, wherein the pores comprise an open distal end, wherein the open distal pore end allows passage of fluid through the pore.

46. The association device of claim 1, wherein substantially only the pore surfaces comprise an association surface.

47. The association device of claim 1, wherein the distance between the nucleic acid probe and the charged surface is no more than about 100 angstroms, about 50 angstroms, about 30 angstroms, or about 20 angstroms.

48. The association device of claim 1, wherein the nucleic acid or the polypeptide probe is at least about 11 to about 20 residues in length.

49. The association device of claim 1, wherein the nucleic acid or polypeptide probes are covalently attached to the association surface.

50. The association device of claim 1, wherein the association surface comprises a streptavidin or streptavidin derivative and the association surface has a net positive (cationic) charge density at a pH lower than pH 5.5 and the association surface has a net negative charge density at a pH higher than pH 5.5.

51. The association device of claim 1, wherein the association surface comprises a histidine or a histidine derivative and the association surface has a net positive (cationic) charge density at a pH lower than about pH 6.7 and the association surface has a net negative charge density at a pH higher than about pH 6.7.

52. The association device of claim 1, wherein the association surface comprises an imidazole or an imidazole derivative and the association surface has a net

positive (cati[redacted]) charge density at a pH lower than about pH[redacted] and the association surface has a net negative charge density at a pH higher than about pH 6.0.

53. The association device of claim 1, wherein the association surface comprises a citrate or a citrate derivative or a carboxylic acid.

54. The association device of claim 1, wherein the association surface comprises an amino acid or peptide linked to the solid substrate surface by its amino terminal end and an aminated oligonucleotide linked to the carboxy terminal end of the amino acid or peptide.

55. The association device of claim 54, wherein the peptide comprises $[(arg)_n\text{-pro}]_n\text{-arg}_n$, $[(arg)_n\text{-pro-gly}]_n\text{-arg}_n$, or $[(arg)_n\text{-gly-gly}]_n\text{-arg}_n$, wherein n is the integer 2, 3, 4, 5 or 6.

56. The association device of claim 54, wherein the peptide comprises $[(arg)_5\text{-pro}]_5\text{-arg}_5$, $[(arg)_5\text{-pro}]_4\text{-arg}_5$, $[(arg)_5\text{-pro-gly}]_3\text{-arg}_5$, $[(arg)_5\text{-pro-gly}]_4\text{-arg}_5$, $[(arg)_5\text{-gly-gly}]_3\text{-arg}_5$, or $[(arg)_5\text{-gly-gly}]4\text{-arg}_5$.

57. A method for associating a nucleic acid or a polypeptide in a sample to a nucleic acid or a polypeptide probe comprising the following steps:

(a) providing a test sample comprising an aqueous solution comprising a nucleic acid or a polypeptide or a combination thereof,

(b) providing an association device as set forth in claim 1; and

(c) contacting the test sample of step (a) with the association device of step (b) under contacting conditions comprising a pH higher or lower than the pI of the association surface, thereby inducing a net positive or net negative charge density, respectively, on the association surface.

5 The method of claim 57, wherein the conta[redacted] conditions comprising a pH higher or lower than the pI of the association surface are established before contacting the test sample of step (a) with the association device of step (b).

59. The method of claim 57, wherein a desired pH is established by contacting the device with an aqueous solution buffered to obtain the desired pH.

60. The method of claim 57, wherein the contacting conditions comprise a pH lower than the pI of the association device to induce sufficient net positive charge density on the association surface to generate a net positive charge density in the pore of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of negatively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of positively charged molecules out of the pore space.

61. The method of claim 60, wherein the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides into the pore space from the aqueous solution outside of the pores.

62. The method of claim 57, wherein the contacting conditions comprise a pH higher than the pI of the association device to induce sufficient net negative charge density on the association surface to generate a net negative charge density in the pore of the device, thereby generating a thermodynamic partitioning equilibrium favorable to the movement of positively charged molecules into the pore space from the aqueous solution outside of the pores and favorable to the movement of negatively charged molecules out of the pore space.

63. The method of claim 62, wherein the thermodynamic partitioning equilibrium is favorable to the movement of negatively charged DNA or RNA polynucleotides or negatively charged polypeptides outside of the pores.

64. The method of claim 57 further comprising removing a nucleic acid or a polypeptide not associated with the probe of step (b) by washing with a buffered aqueous solution.

65. The method of claim 64, wherein the wash conditions induce or maintain a net positive charge density on the surface of the device if the contacting of step (c) comprised net positive charge density conditions, or, wherein the wash conditions induce or maintain a net negative charge density on the surface of the device if the contacting of step (c) comprised net negative charge density conditions.

66. The method of claim 64 further comprising detecting the nucleic acid or polypeptide remaining associated with the nucleic acid or polypeptide probe after the washing and removal of non-associated sample nucleic acid or polypeptide.

67. The method of claim 64 further comprising removing the nucleic acid or polypeptide by washing under conditions comprising a pH higher than the pI of the association surface if the contacting conditions of step (c) comprised a pH lower than the pI of the association surface, or, by washing in an aqueous solution comprising a pH lower than pI of the association surface if the contacting conditions of step (c) comprised a pH higher than the pI of the association surface.

68. A method for detecting at least a single base pair difference between a nucleic acid in a test sample and an oligonucleotide probe comprising the following steps:

(a) providing a test sample comprising a nucleic acid;

(b) providing an association device as set forth in claim 1;

(c) [redacted]ntacting the test sample of step (a) with the ation device of step (b) under conditions that induce the substrate surface or the association surface to have a net positive (cationic) charge density under no salt or low salt conditions;

(d) altering the association surface to an anionic environment by changing the conditions to comprise a pH that induces a net negative (anionic) charge density to the association surface, or, a neutral charge density by coating the association surface with a neutral or anionic polymer composition;

(e) removing test sample nucleic acid not associated with a probe of the device of step (b) under the altered conditions of step (d); and

(f) detecting nucleic acid remaining hybridized to the nucleic acid probe after step (e).

69. A method for making a hybridization device comprising an oligonucleotide probe linked to a solid substrate comprising the following steps:

(a) providing a clean porous polystyrene surface or equivalent;

(b) providing a biotinylated nucleic acid probe;

(c) providing a strepavidin tetramer;

(d) contacting the biotinylated nucleic acid probe of step (b) with the strepavidin tetramer of step (c) in an aqueous solution;

(e) applying the aqueous solution of step (d) directly to the polystyrene surface of step (a);

(f) incubating the probe-applied polystyrene of step (e) in a humid environment for a sufficient amount of time to allow stable absorption of the strepavidin to the polystyrene surface.

70. A method for making a porous surface of an association device comprising an oligonucleotide or polypeptide probe linked to a solid substrate, wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end opening to the external

surface to allow passage of fluid into the pores, and wherein the pore surfaces comprise an association surface, comprising:

(a) co-polymerizing streptaviden and biotinylated nucleic acid probes into a mixture of acrylamide and bisacrylamide, under conditions wherein a porous matrix polymerizes; or,

(b) co-polymerizing streptaviden into a mixture of acrylamide and bisacrylamide, under conditions wherein a porous matrix polymerizes, and, after polymerization, adding biotinylated nucleic acid probe to the polymerized porous matrix by perfusion.

71. The method of claim 70, the mixture in step (a) or step (b) comprises about 19% acrylamide and about 1% bisacrylamide.

72. The method of claim 70, wherein the final concentration of streptaviden in the mixture of step (a) or step (b) is about 10^{-6} M streptaviden tetramer

73. A method for making a porous surface of an association device comprising an oligonucleotide or polypeptide probe linked to a solid substrate, wherein the solid substrate comprises a substrate surface comprising an external substrate surface and a plurality of internal pores, wherein the pores comprise a proximal end opening to the external surface to allow passage of fluid into the pores, and wherein the pore surfaces comprise an association surface, comprising the following steps:

(a) providing a porous silica matrix;

(b) providing a solution of activated silane;

(c) contacting the porous silica matrix of step (a) with the activated silane of step (b) by a gas phase or fluid phase deposition.

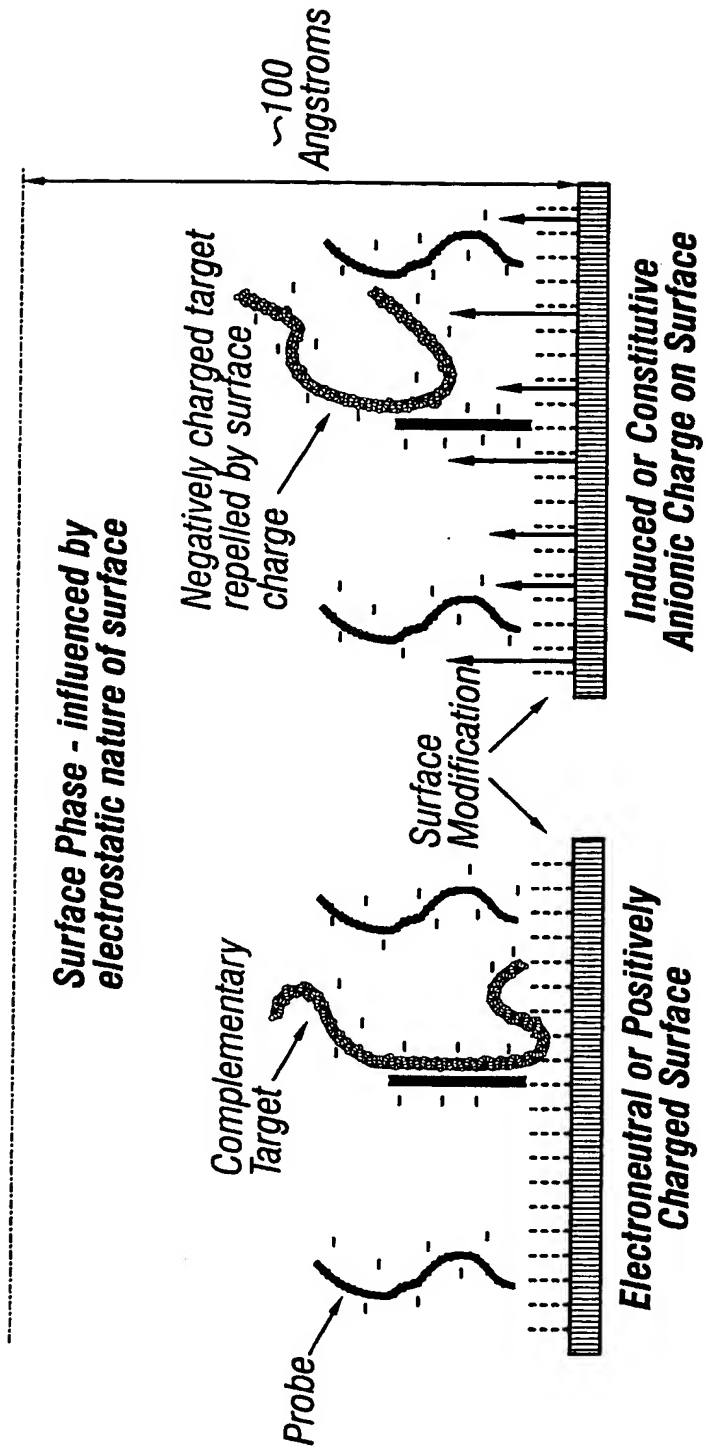

~100 Angstroms
Surface Phase - influenced by electrostatic nature of surface
Negatively charged target repelled by surface charge
Induced or Constitutive Anionic Charge on Surface
Surface Modification
Complementary Target
Electroneutral or Positively Charged Surface
Probe

FIG. 1

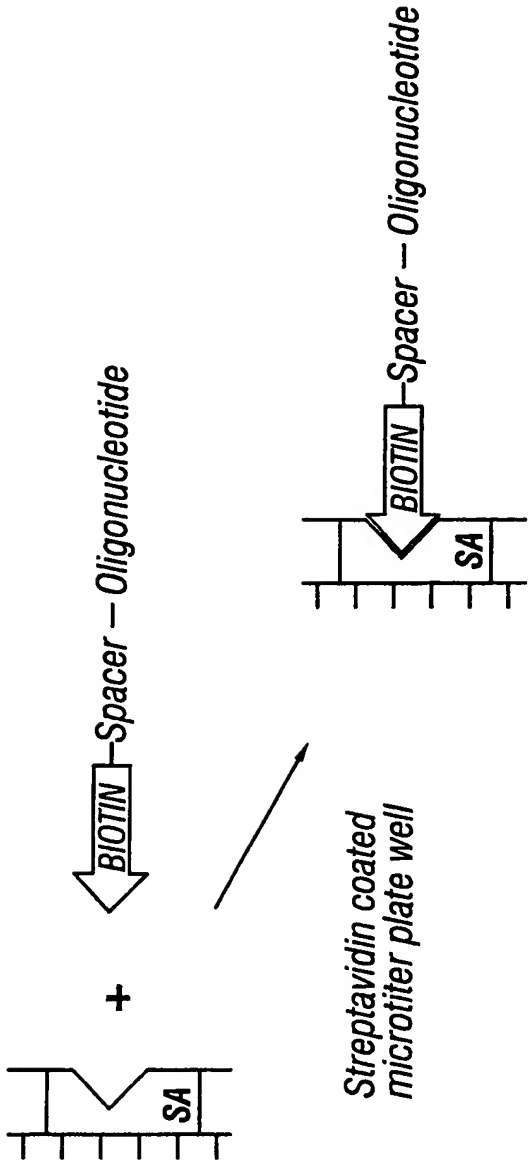

BIOTIN
Spacer – Oligonucleotide
+
SA
Streptavidin coated
microtiter plate well
BIOTIN
Spacer – Oligonucleotide
SA

FIG. 2A

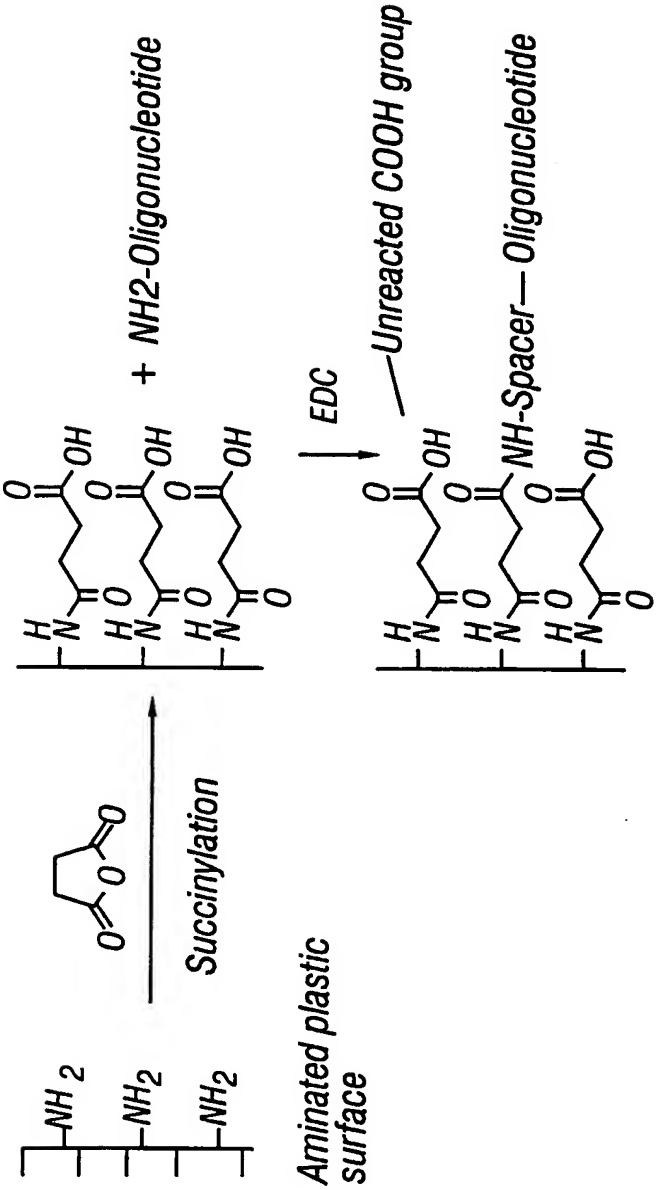

Aminated plastic surface
Succinylation
EDC
+ NH2-Oligonucleotide
—Unreacted COOH group
NH-Spacer— Oligonucleotide

FIG. 2B

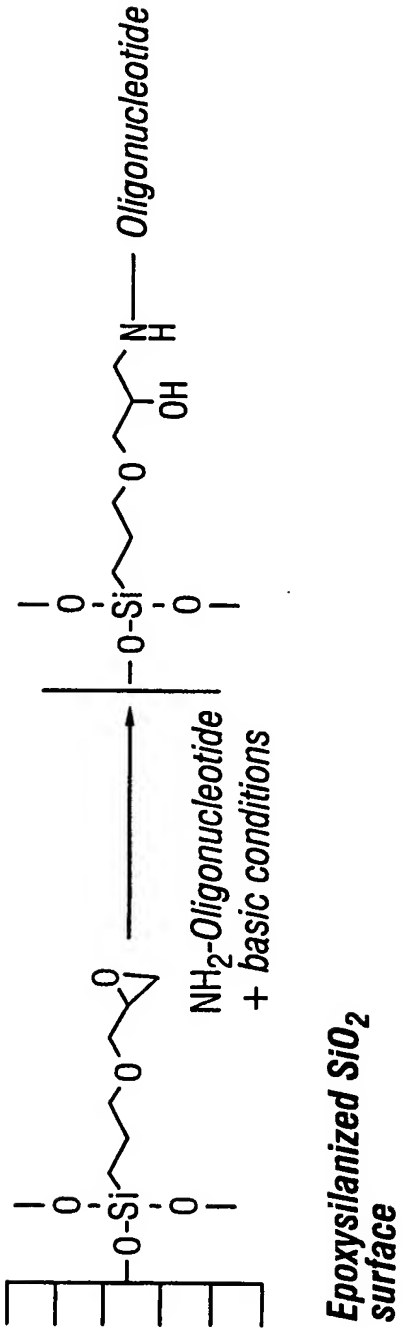

Oligonucleotide
N
H
OH
O
O-Si-O
O-Si-O
O
O
NH_2-Oligonucleotide
+ basic conditions
Epoxysilanized SiO_2
surface

FIG. 2C

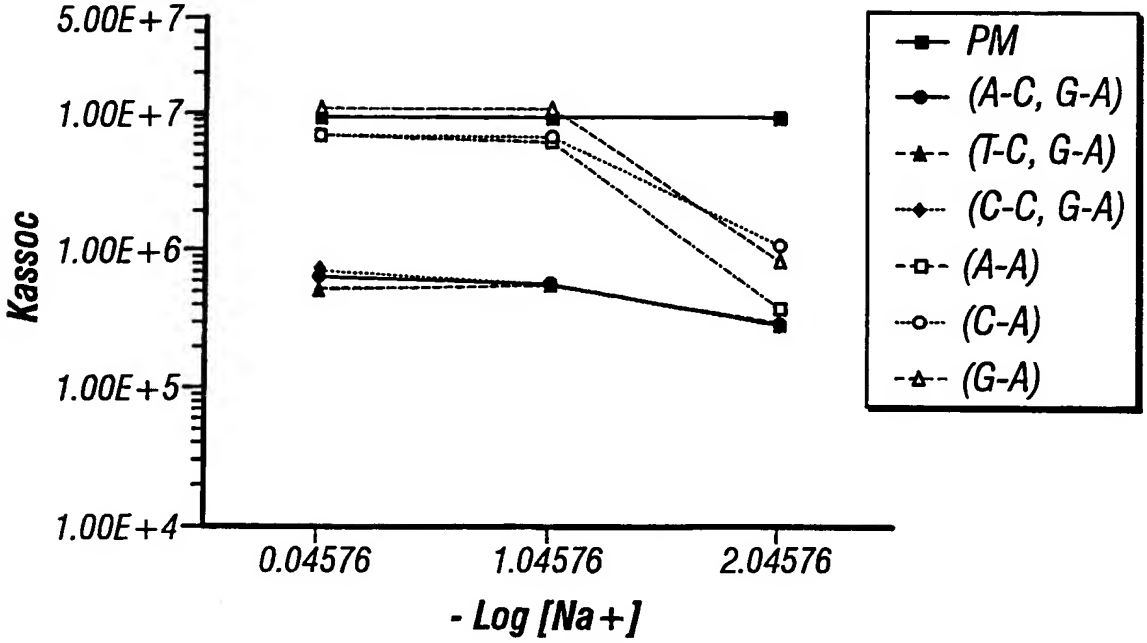

5.00E+7
1.00E+7
1.00E+6
1.00E+5
1.00E+4
Kassoc
0.04576
1.04576
2.04576
- Log [Na+]
PM
(A-C, G-A)
(T-C, G-A)
(C-C, G-A)
(A-A)
(C-A)
(G-A)

FIG. 3

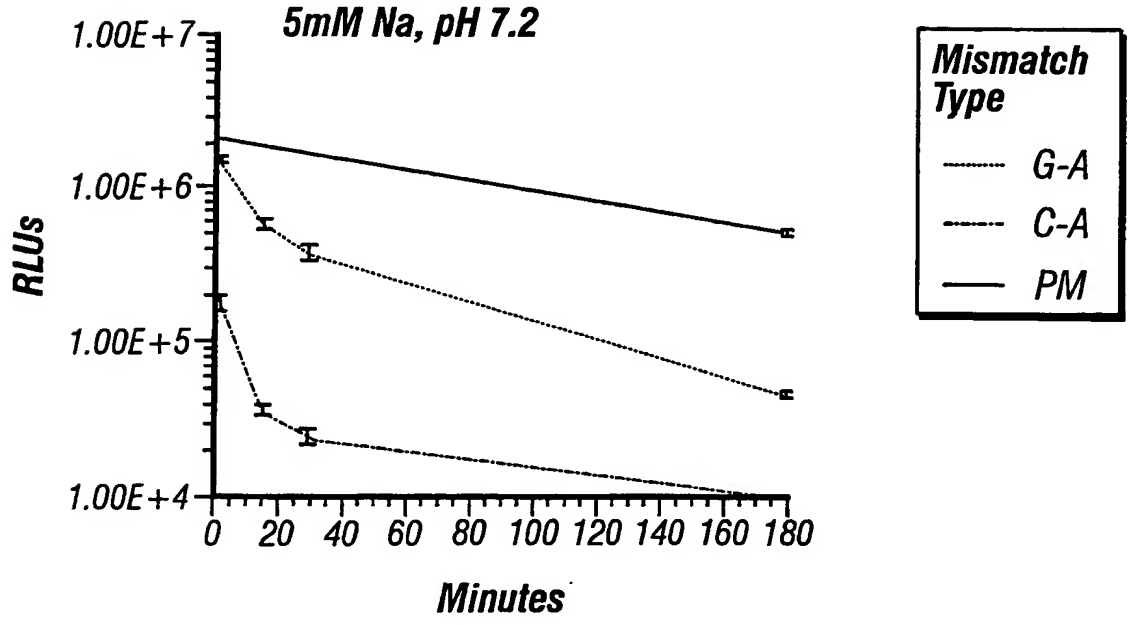

5mM Na, pH 7.2
1.00E+7
1.00E+6
1.00E+5
1.00E+4
RLUs
0 20 40 60 80 100 120 140 160 180
Minutes
Mismatch Type
G-A
C-A
PM

FIG. 4A

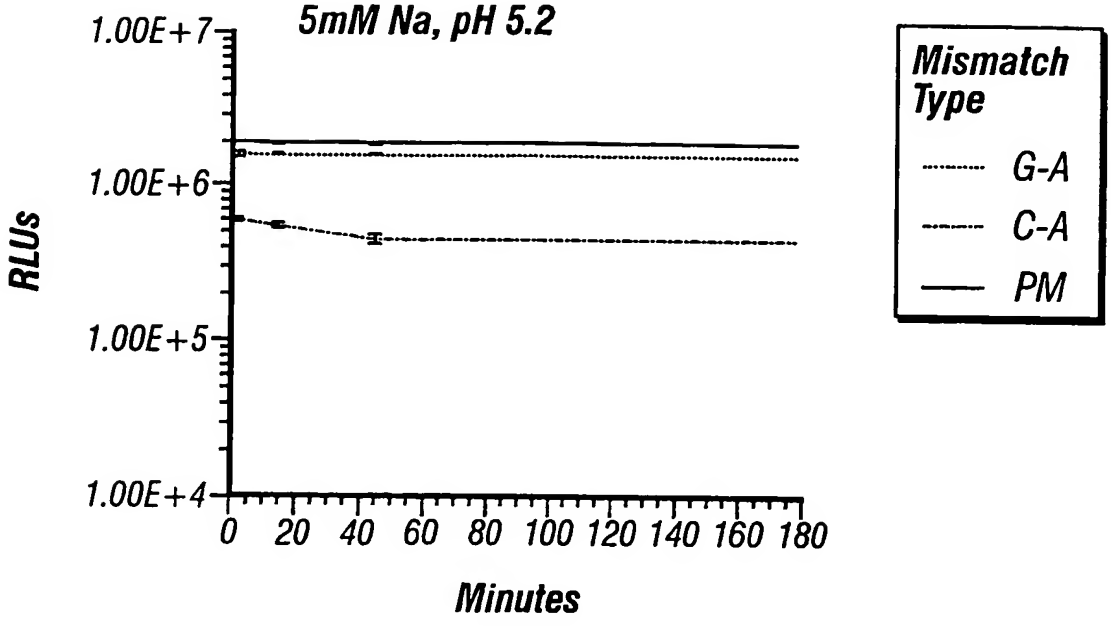

5mM Na, pH 5.2
1.00E+7
1.00E+6
1.00E+5
1.00E+4
RLUs
0 20 40 60 80 100 120 140 160 180
Minutes
Mismatch Type
G-A
C-A
PM

FIG. 4B

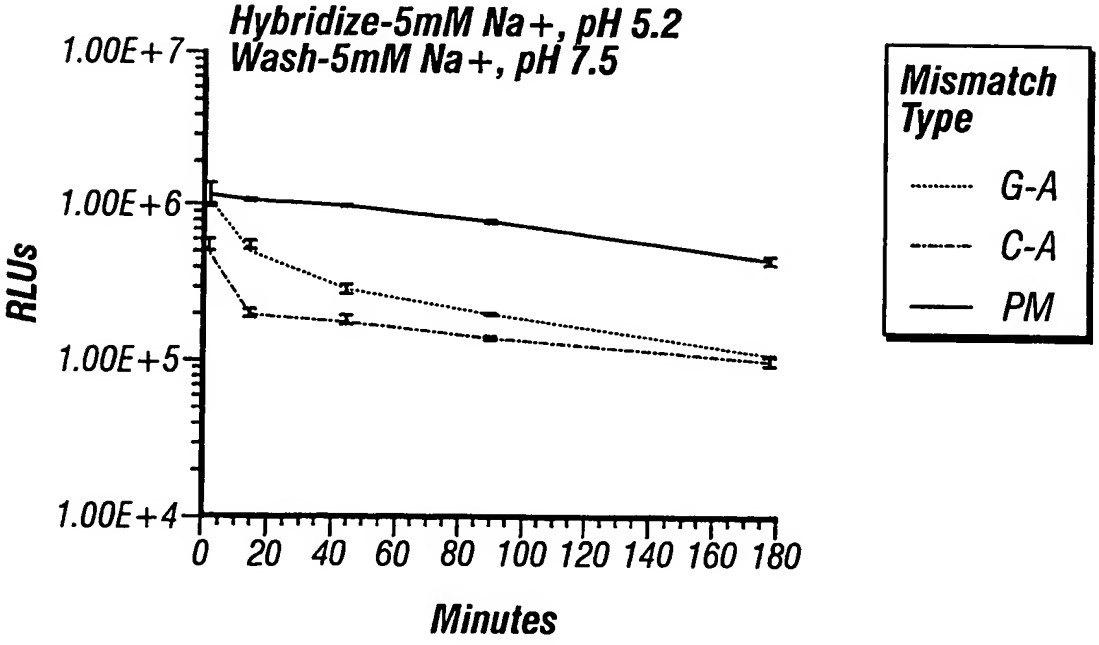

Hybridize-5mM Na+, pH 5.2
Wash-5mM Na+, pH 7.5
1.00E+7
1.00E+6
1.00E+5
1.00E+4
RLUs
0 20 40 60 80 100 120 140 160 180
Minutes
Mismatch Type
G-A
C-A
PM

FIG. 5A

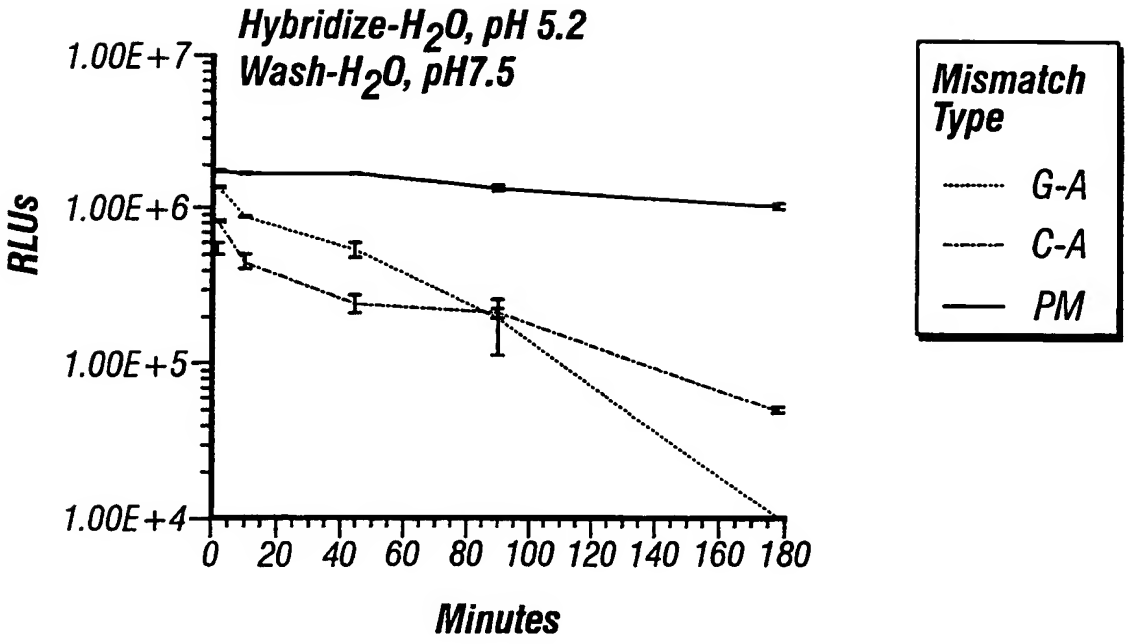

Hybridize-H2O, pH 5.2
Wash-H2O, pH7.5
1.00E+7
1.00E+6
1.00E+5
1.00E+4
RLUs
0 20 40 60 80 100 120 140 160 180
Minutes
Mismatch Type
G-A
C-A
PM

FIG. 5B

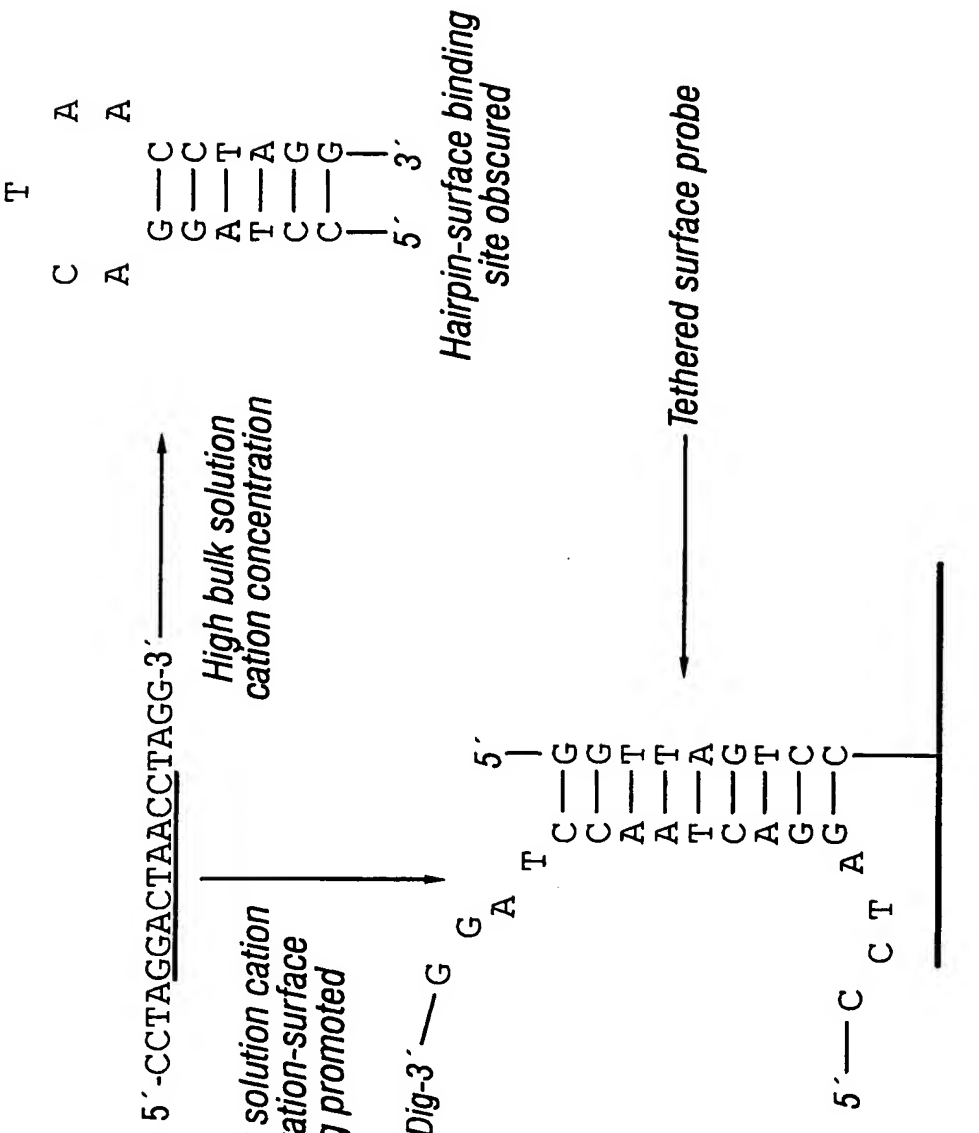
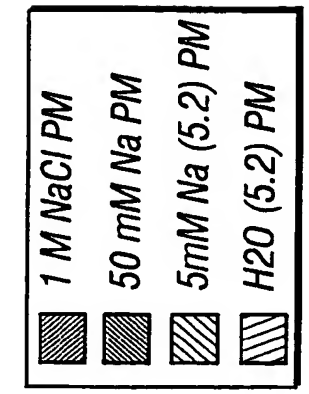

5´-CCTAGGACTAACCTAGG-3´
Lower bulk solution cation concentration-surface binding promoted
High bulk solution cation concentration
Hairpin-surface binding site obscured
Tethered surface probe
Dig-3´
5´
3´

FIG. 6


1 M NaCl PM
50 mM Na PM
5mM Na (5.2) PM
H2O (5.2) PM

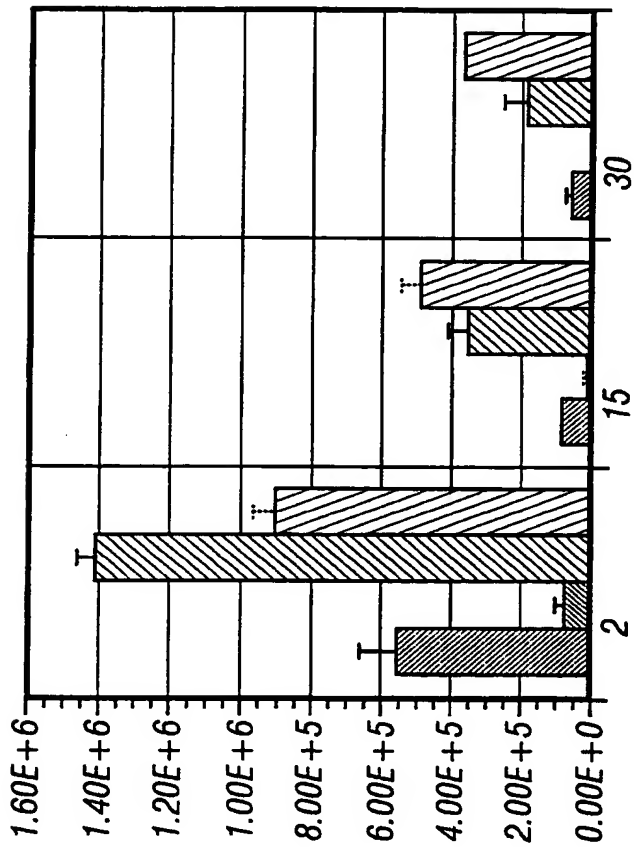
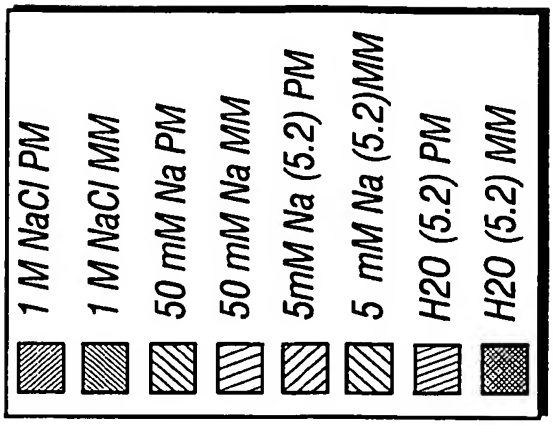

1.60E+6
1.40E+6
1.20E+6
1.00E+6
8.00E+5
6.00E+5
4.00E+5
2.00E+5
0.00E+0
2
15
30
Washing time (minutes)

FIG. 7A


1 M NaCl PM
1 M NaCl MM
50 mM Na PM
50 mM Na MM
5mM Na (5.2) PM
5 mM Na (5.2)MM
H2O (5.2) PM
H2O (5.2) MM

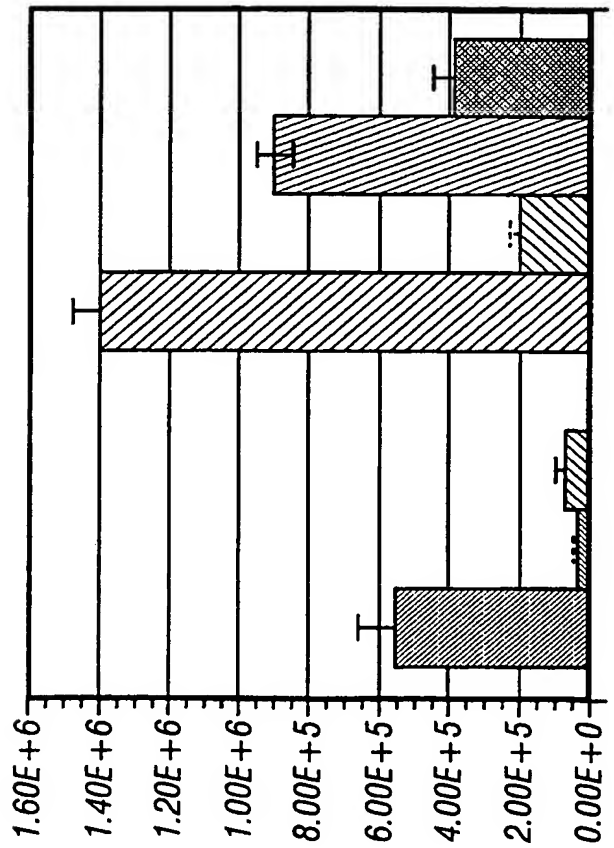

1.60E+6
1.40E+6
1.20E+6
1.00E+6
8.00E+5
6.00E+5
4.00E+5
2.00E+5
0.00E+0

FIG. 7B

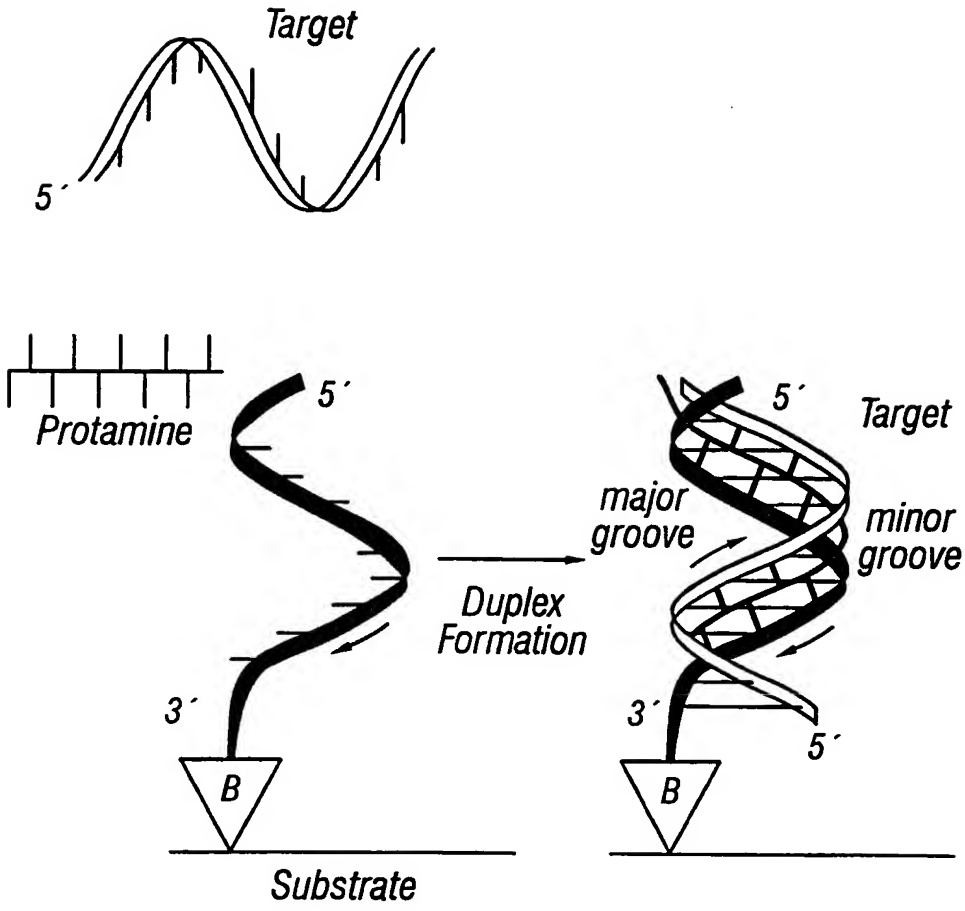

Target
5´
Protamine
5´
3´
B
Substrate
Duplex
Formation
5´
Target
major
groove
minor
groove
3´
5´
B

FIG. 8

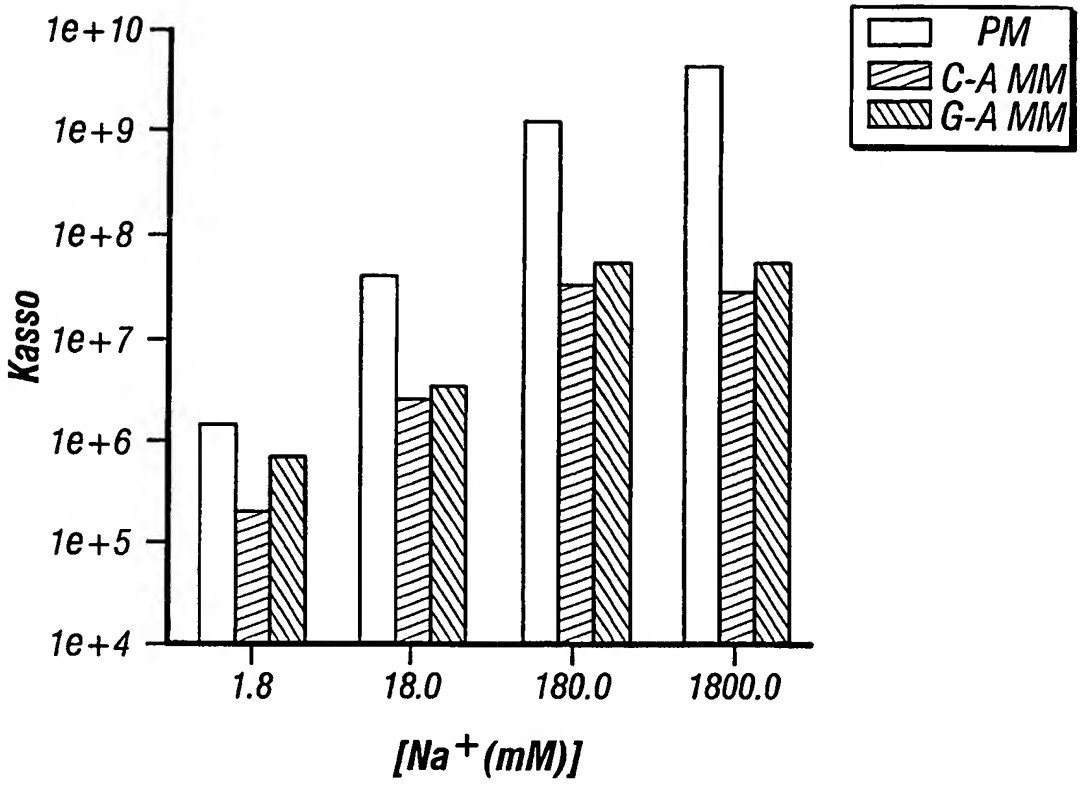

1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
Kasso
1.8
18.0
180.0
1800.0
[Na+(mM)]
PM
C-A MM
G-A MM

FIG. 9A

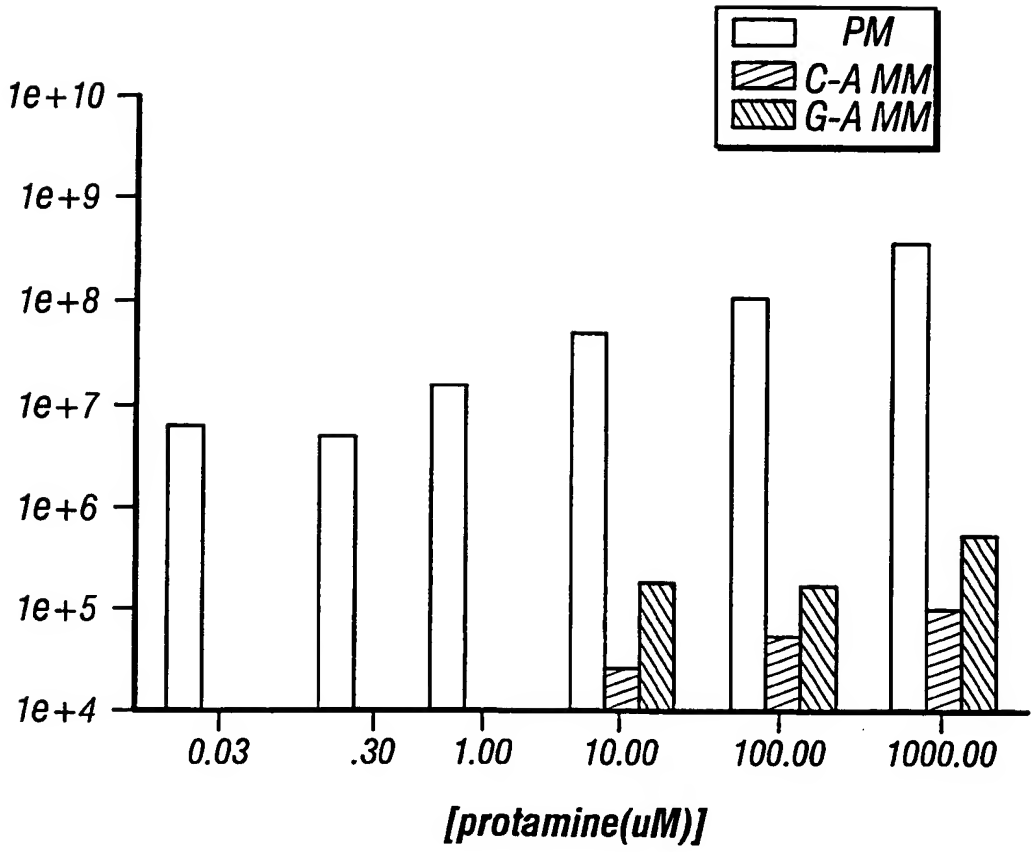

PM
C-A MM
G-A MM
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
0.03
.30
1.00
10.00
100.00
1000.00
[protamine(uM)]

FIG. 9B

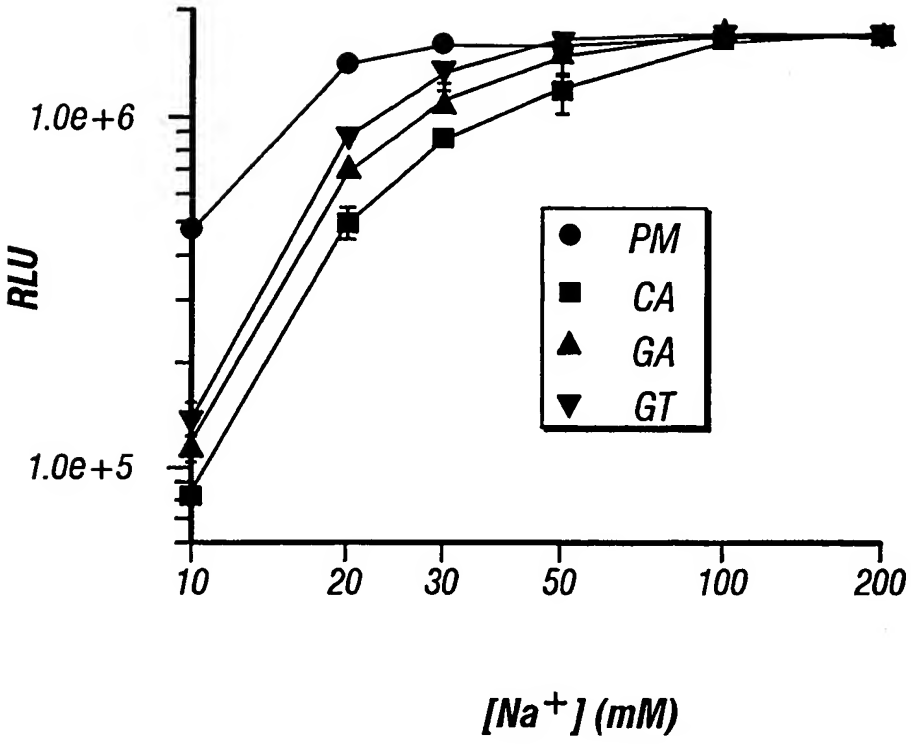


FIG. 10

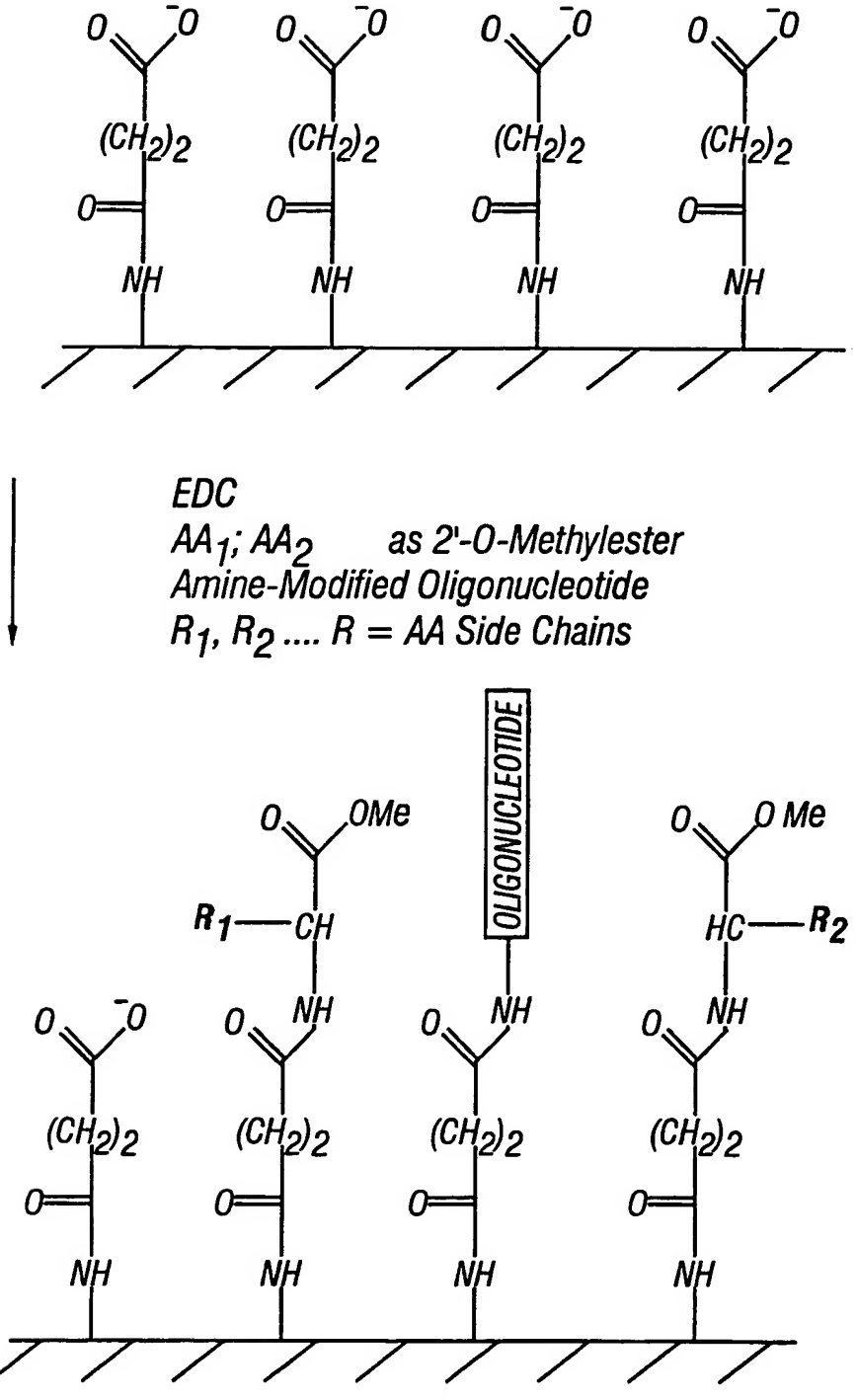

EDC
AA1; AA2 as 2'-O-Methylester
Amine-Modified Oligonucleotide
R1, R2 .... R = AA Side Chains
OLIGONUCLEOTIDE
OMe
O Me
R1—CH
HC—R2
NH
(CH2)2

FIG. 11A

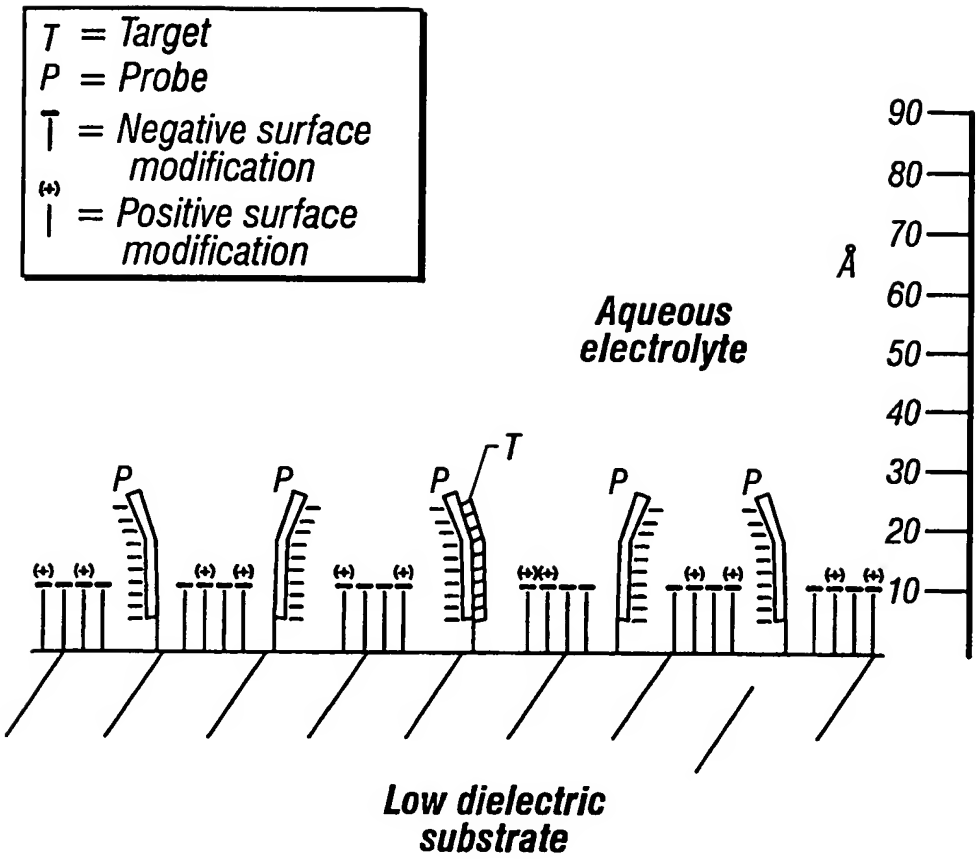

T = Target
P = Probe
= Negative surface modification
= Positive surface modification
90
80
70
Å
60
50
40
30
20
10
Aqueous electrolyte
T
P
P
P
P
P
Low dielectric substrate

FIG. 11B

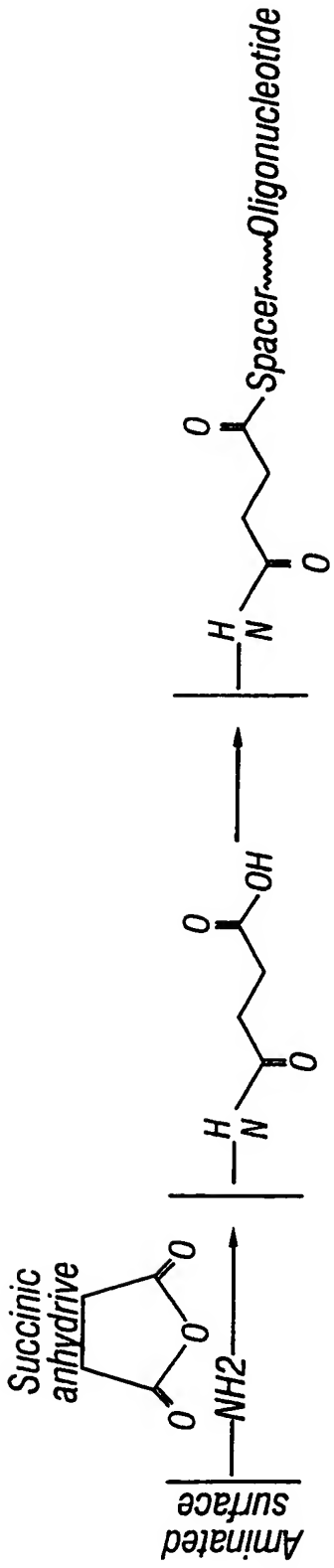

Aminated surface
NH2
Succinic anhydrive
O
O
O
H
N
O
OH
O
H
N
O
O
Spacer
Oligonucleotide

FIG. 12

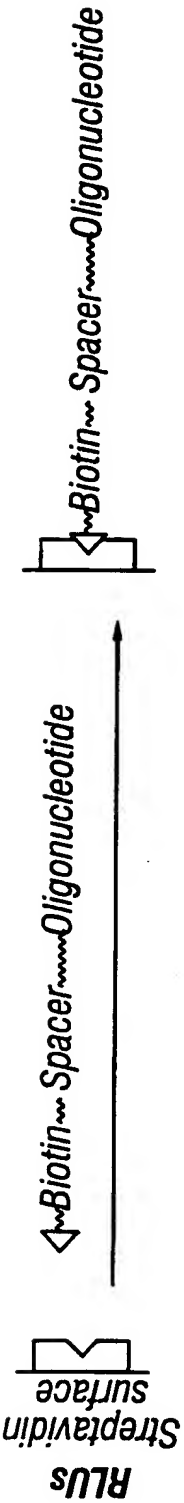

RLUs
Streptavidin surface
Biotin
Spacer
Oligonucleotide
Biotin
Spacer
Oligonucleotide

FIG. 13

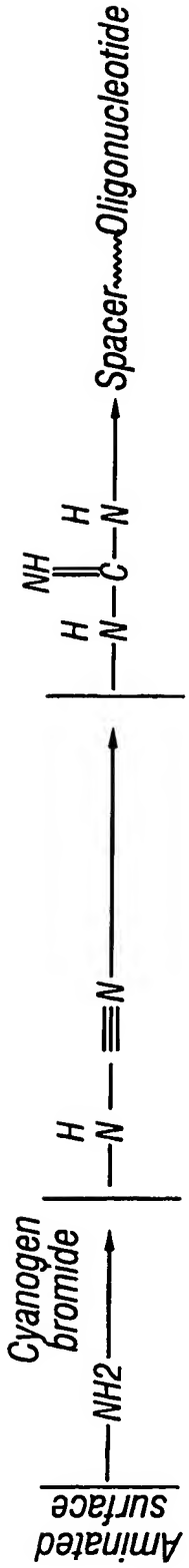

Aminated surface
NH2
Cyanogen bromide
H
N
N
NH
H
N
C
N
Spacer~~Oligonucleotide

FIG. 14


Aminated surface
NH2
α-Haloacids
H
N
CH2COOH
H
N
CH2CO
N
Oligonucleotide

FIG. 15

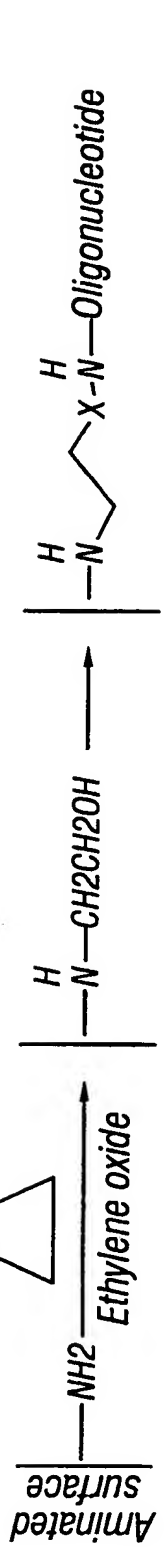

Aminated surface
NH2
Ethylene oxide
O
H
N
CH2CH2OH
H
N
X-N
H
Oligonucleotide

FIG. 16

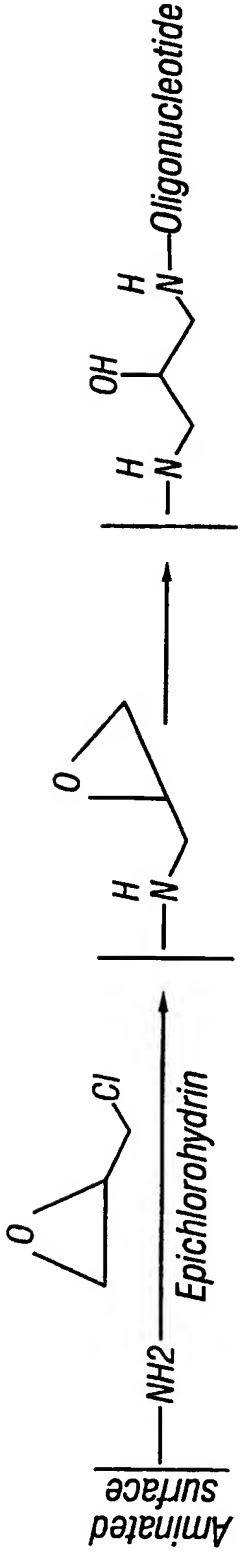

Aminated surface
NH2
Epichlorohydrin
O
Cl
H
N
O
H
N
OH
H
N
Oligonucleotide

FIG. 17

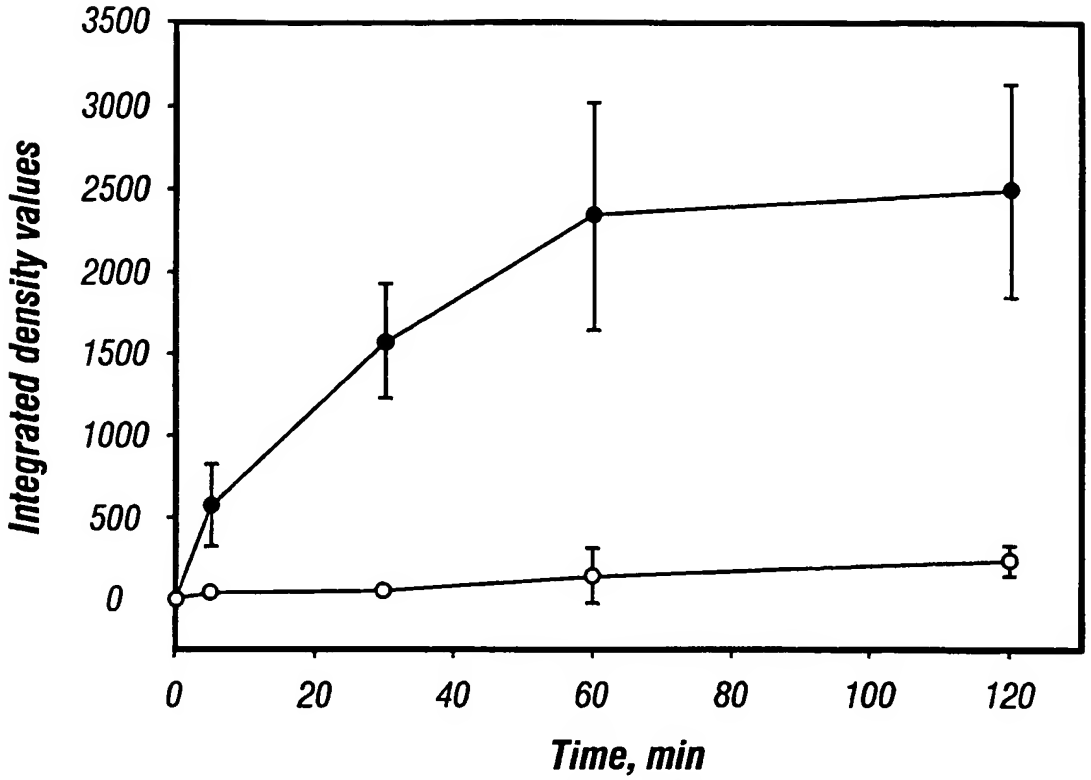


FIG. 18A

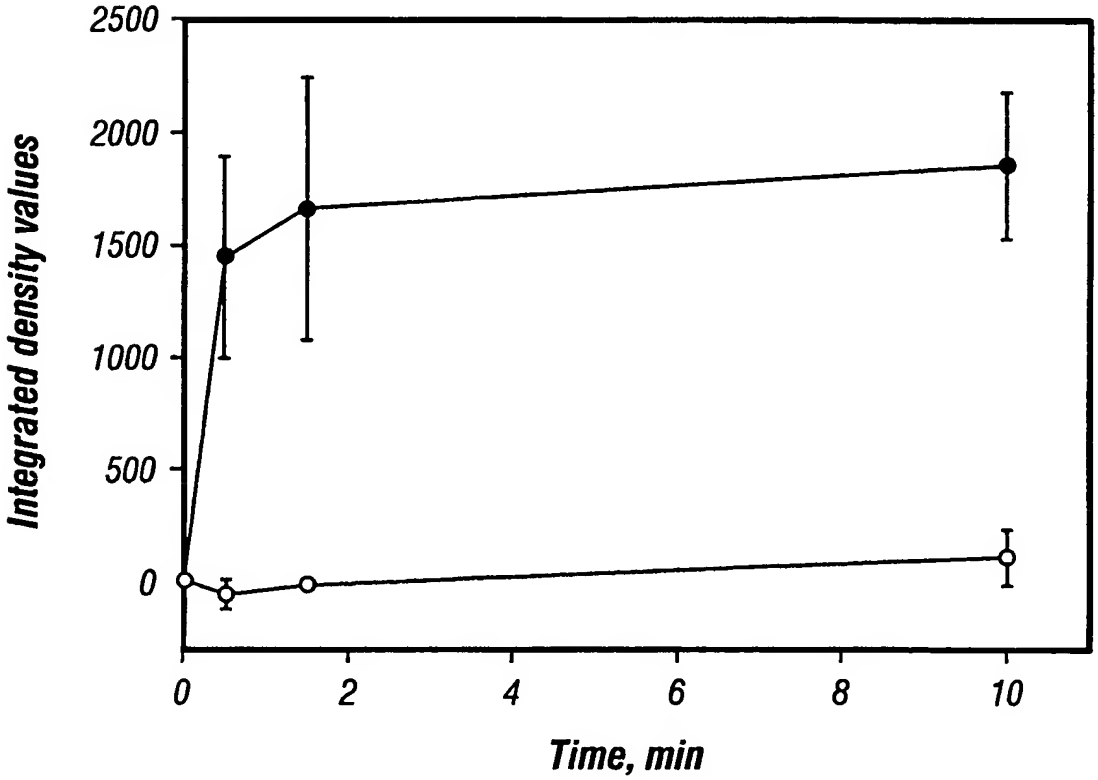


FIG. 18B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/23438

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12M 1/34; G01N 33/00
US CL : 435/287.2; 436/94

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 183, 287.2; 436/501, 94; 536/23.1, 24.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/18226 A1 (BAYLOR COLLEGE OF MEDICINE) 22 May 1997, see entire document.	1-73
Y	US 5,763,175 A (BRENNER) 09 June 1998, see entire document.	1-73
Y	US 5,667,667 A (SOUTHERN) 16 September 1997, see entire document.	1-73
Y	US 5,780,231 A (BRENNER) 14 July 1998, see entire document.	1-73
Y	US 5,962,228 A (BRENNER) 05 October 1999, se entire document.	1-73
T,P	US 6,103,463 A (CHETVERIN et al.) 15 August 2000, see entire document.	1-73

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 SEPTEMBER 2000	Date of mailing of the international search report 18 OCT 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRADLEY L. SISSON Telephone No. (703) 308-0196

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